

Fabricating functionally superior starch-based products with fractionated *Plantago* seed mucilage

Lucija Štrkalj

MSc Nutrition Science

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¹Division of Food, Nutrition and Dietetics,
School of Biosciences, University of Nottingham

²School of Agriculture, Food and Wine,
Faculty of Sciences, The University of Adelaide

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List of proposed publications, in order of future submission

1. Štrkalj, L., Burton, R. A., Yakubov, G. E., Cowley, J. M. (unpublished) Structural and functional properties of fibre from psyllium (*P. ovata*) husk: current knowledge and strategies to expand its application in food and beyond. Target journal: Comprehensive reviews in food science and food safety
2. Štrkalj, L., Yakubov, G. E., Burton, R. A., Cowley, J. M. (unpublished) Fibre fractions from *Plantago ovata* mucilage show contrasting effects on structure and digestibility of corn starch with varying amylose content at different temperature treatments. Target journal: Food & function
3. Štrkalj, L., Burton, R. A., Cowley, J. M., Yakubov, G. E. (unpublished): Competitive gelation between starches and psyllium fibre (*Plantago ovata* mucilage): the effect of composite structure on rheology and freeze-thaw stability. Target journal: Food Hydrocolloids
4. Štrkalj, L., Yakubov, G. E., Burton, R. A., Cowley, J. M. (unpublished) Extraction, composition, and rheology of fibre fractions from four *Plantago* species with varying hydration and gelling profiles. Target journal: Carbohydrate polymers
5. Štrkalj, L., Yakubov, G. E., Burton, R. A., Cowley, J. M. (unpublished) Rheological characterization and enzymatic hydrolysis of composite rice starch-*Plantago* fibre gels. Target journal: Starch/Stärke
6. Štrkalj, L., Hirt, W.J., Yakubov, G. E., Burton, R. A., Cowley, J. M. (unpublished) Application of *P. ovata* and *P. turrifera* fibre in gluten-free bread reveals protective effect on storage stability, but differences in bread quality. Target journal: Food & function

List of presentations

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Additional publications

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CHAPTER 1

Thesis introduction

Starch plays a crucial role in nutrition and food production, serving as a primary source of energy in diets worldwide. Foods like potatoes, rice, and whole grains represent the base of a meal, serve as carbohydrate source, and contribute to overall health by promoting satiety and aiding digestion. Furthermore, starches are versatile in food production; they serve as thickening agents in cooking and because their behaviour varies widely in nature and they are conducive to further modifications, starches are integral to various processing applications beyond food, including pharmaceuticals and paper manufacturing. Due to its relatively low price, it is easily accessible around the globe, and its importance is studied from a culinary aspect, as well as health and metabolism. Despite its significance, starch consumption brings certain challenges. Firstly, starch and starch-based products are usually *over-*processed and lack fibre which is essential for metabolic health. Fibre can reduce blood glucose spikes (a consequence of eating simple carbohydrates and starch), act as prebiotics to maintain gut microbiota, reduce cholesterol and associated risk of cardiovascular illness, and fibre contributes to satiety and healthy eating habits. The addition of fibre and hydrocolloids in starch has long been a subject of research, and improvement of functional properties has been observed by measuring physico-chemical and functional properties, such as digestibility (*in vitro* and *in vivo*), texture analysis, thermal analysis, microstructure, rheology, pasting properties, and many more. Elucidating mechanisms of interaction between starch and fibre is extremely important as it helps to rationalize their use. Therefore, the aim of this thesis was to explore the addition of novel fibre materials to different types of starch, namely fractionated fibre from seeds of the *Plantago* genus. The most utilised and commercially relevant *Plantago* species is *Plantago ovata*, also known as psyllium. *P. ovata* is myxospermous plant, meaning that its seeds produce a gel-like coating called mucilage when wet. Importantly, in its dry form, *P. ovata* mucilage is essentially a layer of dried fibre that can be mechanically separated from the seed. Psyllium husk is often used as a fibre addition to many product categories but its functionality is fairly narrow which has led researchers to explore novel processing methods. Simple fractionations, where the mucilage polysaccharides are extracted with sequential steps usually involving temperature solubilisation and simple separation methods, have gained research interest as they can produce fibres with varying functional

properties from the same starting material. Furthermore, the *Plantago* genus has over 200 species, many of them myxospermous, though underexplored, which prompted us to raise and answer certain research questions. Are there fibre fractions from other *Plantago* species which are comparable to *P. ovata*, and can we utilise their gel properties? How would their rheology differ? Would their addition in certain starches change the overall starch profile? Would those changes be transferable to food products? These questions helped shape topics and the flow of this thesis, which is presented in Fig. 1.1.

Chapter 2, a comprehensive literature review, brings an overview of nutritional and health benefits of *P. ovata* husk, as well as detailed summaries of extraction methods and rheological characterization. Challenges of psyllium husk application are described, which often coincide with the benefits of its high viscosity and proposed five solutions to said challenges with high level rationale.

Chapter 3 focuses on characterization of fibre fractions from four *Plantago* species (*P. ovata*, *P. lanceolata*, *P. turrifera*, *P. drummondii*), as one of the solutions proposed in Chapter 2 was to exploit natural variation in *Plantago*, and study the fractionation behaviour of underexplored species to discover additives with even broader functionality than could be gained from fractionating *P. ovata* alone. We compared corresponding fractions from each species and found that fractions between species shared key chemical similarities but differed in their rheological behaviour. Interestingly, *P. turrifera* and *P. drummondii* produced fractions with extremely high resilience under rheological deformation compared to *P. ovata*, which could be useful in product formulation.

Chapter 4 showed how inclusion of fractions characterised in Chapter 3 affect the quality (colour, freeze-thaw syneresis, rheology, and starch hydrolysis) of rice starch. This study revealed that fibre most likely disrupted the amylose network in gelatinised rice starch, but fibre addition improved viscoelastic properties under stress. Interestingly, *Plantago* fibre fractions increased starch hydrolysis rate in all but one composite gel, which was identified as promising candidate for the improvement of structure and digestibility of starch gels.

Chapter 5, therefore, explores in-depth microscopic and rheological characterization of the promising fraction identified in Chapter 4 when combined with five starches of varying properties at different storage conditions. In this chapter, we propose different interaction mechanisms between *P. ovata* fraction and starches, which were mostly dependent on amylose content of the starch.

Preceding chapters were highly fundamental though we were also interested in exploring fractionations with modifications that might make them more industrially relevant. Chapter 6 presents a one-step fractionation of commercial psyllium husk, where our aim was to study fractionated fibre application and its outcomes which would be industrially relevant. The main finding of this chapter was that the simple fractionation produced two psyllium fractions that had highly contrasting effects on corn starch with varying amylose content, and that those effects were also temperature dependent. This may open the door for adjusting food processing based on viscosity of individual ingredients and texture and digestibility properties of the final product.

Continuing the more application-driven component of this thesis, Chapter 7 investigates the addition of *P. ovata* and *P. turrifera* fibre, identified in Chapters 3 and 4 as having promising rheological differences, in gluten-free rice bread. We studied the effect of *P. ovata* and *P. turrifera* fibre addition on dough behaviour and texture, baking quality of breads, as well as the appearance, texture, storage behaviour of bread and its digestibility in *in vitro* analysis. Our goal was to see if observations from previous chapters would be transferable in complex food products, such as bread, and if the fibre addition improve quality of gluten-free bread. We showed that comparable fractions from alternative *Plantago* species might be suitable alternatives to *P. ovata*, whose supply is becoming more and more volatile.

Finally, Chapter 8, provides an overview of the main findings and avenues for future research.

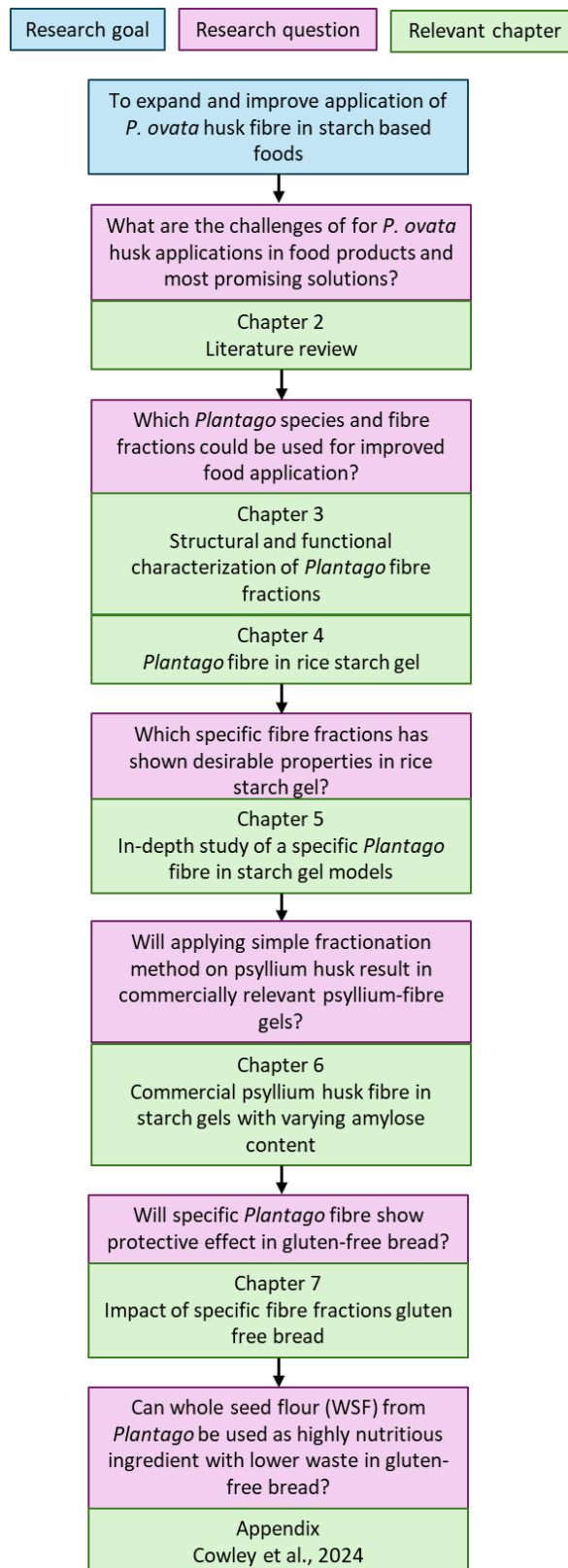


Figure 1. Schematic overview of the research questions that structured the thesis and its relevant research chapters.

CHAPTER 2

Structural and functional properties of fibre from psyllium (*P. ovata*) husk: current knowledge and strategies to expand its application in food and beyond

Title:

Structural and functional properties of fibre from psyllium (*P. ovata*) husk: current knowledge and strategies to expand its application in food and beyond

Authors:

Lucija Strkalj^{1,2}, Rachel A. Burton¹, Gleb E. Yakubov², James M. Cowley^{1*}

¹School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

²Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom

*Corresponding author

Highlights:

- A detailed rheology overview of *Plantago ovata* husk mucilage is presented.
- *P. ovata* husk showed both beneficial and detrimental effects in food products.
- Five strategies are proposed to overcome key limitations in *P. ovata* application.

Abstract:

Psyllium husk fibre, from the milled surface of *Plantago ovata* seeds, is known for its high viscosity and gelling capacity, making it a widespread health supplement with benefits on satiety, glycaemic control, gastrointestinal and cardiovascular health, as well as texturiser, stabiliser, and fat and gluten replacement. Nevertheless, its application often comes with certain challenges, such as difficulties in adjusting hydration level, gumminess, and colour change. The positive and negative results of

the fibre implementation are a consequence of its unique gelation profile and high water uptake. Therefore, a need to uncover the structure and rheological behaviour has yielded detailed studies on its fundamental properties, as well as research with the aim of modifying the behaviour of fibre for the easier application in food products. This review addresses current literature on the nutritional and health benefits of psyllium, rheological characterization, advantages and disadvantages of its application in diverse food groups, and suggests approaches to expand its use by modifying its relatively narrow functionality, such as fractionation, modification, and genetic strategies. Moreover, a new perspective and future direction for research are discussed, which can also be applied to similarly highly viscous fibres. This paper serves as an overview of the fibre's principal and practical properties and connecting insights from multiple avenues of its exploration with the aim of facilitating its expanded use.

Keywords:

Plantago ovata, rheology, fibre nutrition, fractionation, modification

Abbreviations:

A:X = arabinose to xylose; AX = arabinoxylan; AXOS = arabinoxylan oligosaccharides; AEF = alkaline extracted fraction; CWE/CWF = cold water extracted fraction; DMT2= diabetes mellitus type 2; DP = degree of polymerisation; DPPH = 2,2-diphenyl-1-picrylhydrazyl; FC = fibrillated cellulose; GF = gluten free; GI = glycaemic index; HDL = high density lipoprotein; HPH = high pressure homogenization; HWE/HWF = hot water extracted fraction; HX = heteroxylan; IAE = intense agitation extract; LDL = low density lipoprotein; MD = mean dose; SCFA = short chain fatty acids; WAC = water absorbing capacity

1. Introduction

The *Plantago* genus includes over 200 species, of which *Plantago ovata* is the most researched, understood, and commercially important. *P. ovata* is a myxospermous plant, meaning that when its seeds are exposed to water, they produce mucilage – a gel-like coating rich in polysaccharides that has various hypothesised roles in seed protection and dispersal (Cowley & Burton, 2021; Pan et al., 2022). In the case of *P. ovata*, mucilage is derived from the husk – a layer of dried fibre on the seed surface that can be easily removed by stone milling (Phan et al., 2020; Cowley & Burton, 2021). The husk, more commonly known as psyllium husk or, simply, psyllium, is widely available and used as a health supplement and food ingredient. Several literature reviews have been published recently, highlighting the usefulness of psyllium in food products (Belorio & Gómez, 2022), describing a broad range of health benefits (Franco et al., 2020; McRorie et al., 2021), and focusing on the bioactive molecules in *Plantago* plants (Zhang et al., 2019). Specific applications in foods include the use of psyllium as a thickener, emulsion stabiliser, and structuring component with a strong capacity to replicate some functions of gluten. For gluten replacement, the key properties of psyllium are the fibrous nature of its gel network (Ren et al., 2020c) and its hydrocolloid functionality with high water-holding capacity. The addition of psyllium markedly improves the acceptability, storage stability and texture of gluten-free (GF) bread (Fratelli et al., 2021). Thanks to these properties, psyllium is the third most used gluten substitute in the baking industry, after hydroxypropyl methylcellulose (HPMC) and xanthan gum, (Belorio & Gómez, 2022). In addition, psyllium is used to produce edible and biodegradable food packaging and films; it provides high water vapour permeability and can be combined with other desirable materials, such as proteins (Cikrikci Erunsal et al., 2023; Zhang et al., 2020). Psyllium husk is used for improvement of gastrointestinal function, and it is well tolerated by people who have irritable bowel syndrome (A. Alhasani et al., 2023; Gunn et al., 2022; Harris et al., 2023; Jalanka et al., 2019; Major et al., 2018; Moayyedi et al., 2019). Due to its satiety effects, psyllium is used for weight loss and management (Gibb et al., 2023). It also has positive influence on health outcomes on cardiovascular health, including cholesterol levels, and glycaemic control, and is recommended to people with metabolic syndrome (Pal & Radavelli-Bagatini, 2012).

Despite the high demand for psyllium in an ever-increasing number of applications, its growing area has not significantly expanded, leading to volatility in the psyllium supply chain. In recent years, export of psyllium by India, the world's largest producer of psyllium, has fluctuated from 32,000 tons to as high as 56,000 tons per year (10-year standard deviation = 7442 tons) (Govt India Dept. of Commerce, 2024). Despite this, cost per unit (i.e. US \$/ton) has increased by 83% over the last decade and is expected to continue to increase. This coupled with the agronomic constraints and lack of breeding technologies for *P. ovata* highlights the need to further develop the species as a crop and build technologies, knowledge and processes that might make psyllium application more efficient (Cowley & Burton, 2021; Herliana et al., 2023).

In this review, the in-depth characterisation and practical applications of polysaccharides derived from *P. ovata* mucilage are summarised. It is important to note that in the literature, the terms "psyllium", "psyllium husk" and "*P. ovata* husk" are used interchangeably, along with traditional names like ispaghula and isabgol, and the expressions which authors originally used are kept in the review.

Previously published reviews have mostly focussed on applications in food products or health benefits of psyllium husk which, though important, often do not capture the full complexity of psyllium's unique chemistry and functionality nor how the said complexity could be utilised for research and commercial purposes. In this review, the aim was to bridge the gap between understanding of the intricately layered structure of *P. ovata* mucilage and its functionality, highlight the challenges of its implementation, and offer solutions to improve certain aspects of its use in food products.

2. Nutritional and health benefits

The first mention of psyllium for medicinal and pharmaceutical purposes was published in 1927 (Nadkarni, 1927). Since then, numerous studies have reported the beneficial effects of psyllium on cardiovascular and gastrointestinal health. In this review, the focus is on four major health effects of psyllium and how its structural properties determine the underpinning mechanisms. The primary focus of this

section are the mechanisms and pathways that are connected to main health benefits of psyllium (Fig. 1). For more detailed information related to hormone regulations and mechanisms linking the primary biophysical and physiological responses with health benefits, the readers can be referred to several published reviews on this topic (Chen et al., 2022; McRorie et al., 2021).

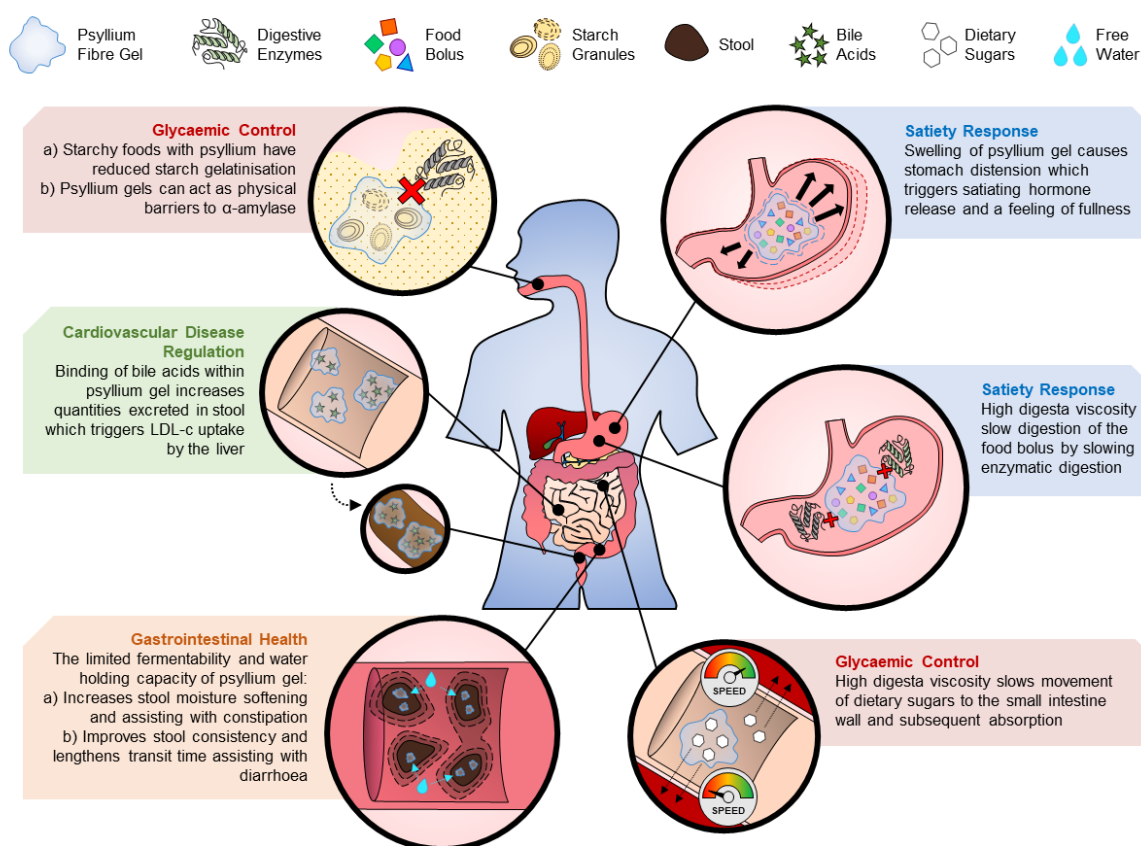


Figure 1. Schematic summary of the dietary fibre-related health benefits of psyllium consumption.

2.1. Satiety control and weight management

Psyllium has a long history as an ingredient or supplement to promote satiety and help with weight control. Its primary effects of viscosification and water absorption are important as they result in increased chyme (the mixture of partly digested food and gastric juice) viscosity, which then triggers a number of mechanisms related to satiety (Pal & Radavelli-Bagatini, 2012). For comparison, 2 g of psyllium/100 g of water has significantly higher viscosity (48.40 Pa·s (Pascal-second)) than oat bran, wheat bran, or rice bran (at 1.36, 0.51, and 0.10 Pa·s, respectively). Moreover, during

simulated digestion (after 4 h of gastric digestion), the viscosity of a psyllium-containing solution was 16.15 Pa·s, while oat bran, wheat bran and rice bran formulations were 0.74, 0.64, and 0.54 Pa·s, respectively (Dikeman et al., 2006; Pavlovich-Abril et al., 2012). It should be noted that psyllium and the aforementioned brans are different in solubility – the brans are not soluble, while psyllium is considered to be soluble (i.e. it forms a gel). However, these are commonly used fibre supplements, and their comparison shows that psyllium has extremely high viscosity which is preserved throughout the simulated digestion. High chyme viscosity slows interactions between enzymes and macronutrients; thus, the macronutrients are less hydrolysed and reach the small intestine in a more intact form. The extended presence of nutrients in the small intestine leads to two physiological outcomes: release of appetite regulating hormones and induction of the ileal brake mechanism (Franco et al., 2020; Kristensen & Jensen, 2011). Appetite regulating hormones, such as cholecystokinin, glucagon-like peptide 1, and peptide YY are released into circulation, acting on the nervous system where they promote feelings of satiety, reducing hunger. The ileal brake mechanism is triggered when higher amounts of undigested nutrients reach the distal ileum. This causes a distal-to-proximal feedback mechanism which slows the transit of a meal through the GI tract to optimise nutrient digestion and absorption (Maljaars et al., 2008). The ileal brake can cause a number of cascade effects, all resulting in the increased small intestinal transit time: reduced jejunal motility, delayed gastric emptying, and reduced secretion of pancreatic enzymes, bile acids, and gastric acid (Maljaars et al., 2008). All these mechanisms contribute to increased satiety and reduced energy intake, which ultimately results in the weight control. When compared to guar gum, psyllium ingestion provided sustained weight loss, and compared to a restrictive diet, showed greater weight loss during a six-month study (Cicero et al., 2010). More precisely, McRorie et al. (2021) have surmised that daily intake of 6.8 to 7.6 g of psyllium could significantly reduce the feelings of hunger. A newly published meta-analysis has found that psyllium taken before meals (mean dose (MD) = 10.8 g/day for 4.8 months) can reduce body weight (MD = -2.1 kg), body mass index (MD = -0.8 kg/m²), and waist circumference (MD = -2.2 cm) in overweight and obese participants (Gibb et al., 2023).

2.2. Cardiovascular health

Cardiovascular diseases (CVD) are the leading cause of death worldwide (Goldsborough et al., 2022), thus it is vital to understand and apply the preventative role of dietary fibre. It has been shown through clinical trials that psyllium supplementation successfully lowers total and low density lipoprotein (LDL) cholesterol (the 'bad' cholesterol associated with atherosclerosis), while not affecting levels of high density lipoprotein (HDL) cholesterol (the 'good' cholesterol that promotes cholesterol excretion) (McRorie et al., 2021). The lowering effect can be explained by two mechanisms: reduced digestion of lipids and increased synthesis of bile acids. Both mechanisms are thought to be related to viscosification. Due to a higher digesta viscosity, lipases and lipids will have decreased interactions resulting in the reduced digestion and absorption. Bile acids are produced by the liver and released into the small intestine (duodenum) where they emulsify lipids and enable their digestion. Around 95% of bile acids are recovered in the terminal ileum and reused for the next digestion cycle. When chyme containing psyllium passes through the small intestine, viscous psyllium gels entrap bile acids, leading to their excretion and therefore they are not reabsorbed and reused. This causes a reduction in LDL cholesterol level in the blood as the liver uses LDL to produce bile acids and thus this positively affects the cardiovascular system (Cai & Chen, 2014). A randomised study compared the impact of the wheat dextrin and psyllium supplementation (3.5 and 3.4 g/day, respectively) in hypercholesterolemic adults over three months (McRorie et al., 2017). The results showed a 17% LDL reduction and a 11% total cholesterol reduction in a subject group given psyllium, whereas the wheat dextrin group showed no significant change. Another study reported a 7.7% decrease in total cholesterol and 10.7% decrease in LDL cholesterol when 7 g of psyllium/day was given to dyslipidaemic children and adolescents (6-19 years) (Ribas et al., 2015). To contextualise how these findings could impact the economy, a cost-benefit analysis concluded that an intake of 7 g psyllium/day in certain patient groups (>45 years, LDL cholesterol level 130 mg/dL) could save, on average, USD 870 million per year in healthcare costs (data for 2013-2020 for the US) (Shanahan et al., 2019). It is evident from the literature that dietary fibre needs to be highly viscous to elicit a cholesterol reducing effect. So far, only two fibres are FDA approved and can have the claim of

reducing risk of CVD by lowering serum cholesterol level: psyllium and β -glucan, both potentially viscosifying, acid-resistant soluble fibres (Food and Drug Administration, 2002).

2.3. Glycaemic control and the effect on starch

Elevated blood glucose level is one of the major risk factors for developing metabolic syndrome, CVD, insulin resistance, and diabetes mellitus type 2 (DMT2). More precisely, postprandial blood glucose level is a better predictor for metabolic syndrome development than the fasting glucose level (Giacco et al., 2016). Taking that into account, it is important to consider the health implications when ingesting carbohydrates, which are the primary macronutrient driving dose-response reactions for blood glucose (Giacco et al., 2016). Implementing psyllium into a diet can help lower both fasting and postprandial blood glucose levels, with the most prominent effect on people with DMT2 or with developing DMT2 (McRorie et al., 2021). As with other health benefits where psyllium's viscosification effect reduces nutrient digestion/absorption, chyme containing psyllium shows slower glucose diffusion and absorption which decreases the postprandial peak. A review published by Pal and Radavelli-Bagatini (2012) has highlighted that psyllium intake of 10–14 g/day for 6–8 weeks reduced glucose levels, but increased intake (21–23 g/day, for at least 12 weeks) reduced plasma insulin levels. However, longer studies (six months) have shown that the lower doses of psyllium (10 g/day) can also decrease both glucose and insulin levels (Cicero et al., 2010). Gibb et al. (2015) concluded that psyllium supplementation can decrease fasting glucose, levels of HbA1c (glycosylated haemoglobin widely used as a biomarker for DMT2), and postprandial glucose in diabetic patients, and psyllium also has a reducing effect on insulin and postprandial glucose in healthy individuals.

Besides its physiological effect, psyllium affects starch behaviour in food processing, which should be applied when developing or improving food formulations (Zhou et al., 2022). Starch is a complex glucose-containing carbohydrate and a diet staple all over the world constituting the most important source of energy for humans. Its digestibility, and glucose release, depends on many factors (such as amylose to amylopectin ratio, type of processing, gelatinization and retrogradation, etc.), but

this discussion focuses on how soluble viscous fibres, such as psyllium, can alter the susceptibility of starch to digestive amylases. (Singh et al., 2010; Zhang et al., 2022). Briefly, the starch granules become more accessible to amylase when they become gelatinised (i.e. undergo hydrothermal treatment) because the ordered crystalline structure of starch granules is degraded. As with other hydrocolloids, combining psyllium with starch or flour in starch-based foods could potentially 1) limit their gelatinisation extent by physically restricting granule swelling, and/or 2) create a network around the starch granules that acts as a mechanical barrier preventing amylase from reaching the granule (Zhang et al., 2022). This was tested in wheat, potato, and tapioca starches (Sevilmis & Sensoy, 2022), and it was shown that psyllium affected structural properties of starches by restricting granule swelling and gelatinization, and affecting the ordered structure of the residual starch after cooking when psyllium was added, and therefore led to a decreased rate of starch digestion, which can be associated with the postprandial peak. When comparing the addition of psyllium and cellulose fibres (both at 10%) in bread and crackers, results showed that psyllium reduced levels of rapidly digested starch and increased slowly digested starch in both products, while cellulose fibres had no significant effect (Bilgic & Sensoy, 2023). Even though these studies employed *in vitro* digestion models, they highlight the importance of viscous fibres in controlling glycaemic index of foods and constitute a factor that should be considered in food processing.

2.4. Gastrointestinal health

It is recommended that the daily intake of fibre for adults should be 30 g or more (per 2000 kcal per day) (McKeown et al., 2022). However, the type of fibre is important when distinguishing its nutritional benefits, as structural properties dictate its functionality and mechanisms through which fibre achieves certain health effects. Fibres are usually categorised as soluble and insoluble, however that does not reflect on their health functionality (Gidley & Yakubov, 2019). Soluble fibre is chemically characterised as soluble or gel-forming in water, but it can be viscous or non-viscous. Fibres that are viscous provide cholesterol lowering and satiety effects (McRorie & McKeown, 2017). Insoluble fibre is not water-soluble, and is not viscous, therefore it does not show health benefits that are related to viscosity (McRorie & McKeown,

2017). Psyllium is a soluble, non-fermentable, highly viscous fibre with a range of benefits and applications targeted towards health of the gastrointestinal tract (Gibb et al., 2023). Psyllium administration is therapeutic toward both constipation and diarrhoea. Psyllium gel has high water-holding capacity which withstands dehydration in the large intestine, increasing stool weight and acting as a softener and emollient, to ease laxation (McRorie & McKeown, 2017). An MRI study in humans showed that psyllium intake (7 g, 3 times a day) increased the water content in the small bowel, colonic volume, and T1 measurement (determination of longitudinal relaxation time which describes the recovery of magnetization along the direction of main magnetic field), which correlates with free water measurement and water content of the stool (Major et al., 2018). In a healthy (non-constipation/diarrhoea) population, 1 g of psyllium increases stool weight by 5.9–6.1 g, which is higher than wheat or oat bran (4.9–5.4 and 3.4–4.5 g, respectively) (Wärnberg et al., 2009). It can be concluded that the laxative effect of soluble fibre (such as psyllium) is a result of increased stool moisture. Conversely, the laxative effect of insoluble fibre (such as cereal brans) is caused by their mechanical irritation effect on bowel mucosa. Mucous membranes then release water, as a defence reaction against the irritation, which increases water content of the stool (McRorie & McKeown, 2017). For patients suffering from diarrhoea, psyllium also offers relief as the high water absorption capacity and increased colon transit time allow for appropriate stool formation (Wärnberg et al., 2009).

Considering its beneficial effect on constipation and diarrhoea, and the gentle nature of its effects (i.e. non-abrasive effect on mucous gut membrane), it is justified that psyllium is recommended therapy for patients with irritable bowel syndrome (IBS), who can experience periods of both constipation and diarrhoea. Per the American College of Gastroenterology, psyllium is the only recommended fibre for IBS and constipation treatment (Ford et al., 2018). It is also recommended by the Japanese Society of Gastroenterology and Canadian Association of Gastroenterology as an IBS treatment (Fukudo et al., 2021; Moayyedi et al., 2019).

Nevertheless, fermentability of psyllium husk and its polysaccharides has been a topic of debate. As mentioned, psyllium is considered to be soluble fibre, and other soluble

fibres are associated with fermentability, such as pectin or guar gum, though the soluble/insoluble definitions are complicated (Gidley & Yakubov, 2019). Indeed, *in vitro* data has indicated psyllium has some level of fermentability (Campbell & Fahey, 1997), and *in vivo* studies in constipated patients have shown productions of short chain fatty acids (SCFAs) and changes in bacterial populations when psyllium was added to a diet (Jalanka et al., 2019). However, current consensus agrees that psyllium is mostly non-fermentable or slowly fermentable due to three reasons: its gel structure prevents bacteria from entering the substrate; substitution density limits backbone access by enzymes; and densely branched structure present steric hindrance to human intestinal and microbiota enzymes (Marlett & Fischer, 2003; McRorie et al., 2021). Marteau et al. (1994) found that psyllium appeared still intact in stools, which contributed to its bulking effect, but it also led to higher amounts of acetate, propionate, and total SCFAs implying that fermentation was elevated. Psyllium consumption (15 g per day, for 12 days) is also shown to increase apparent viscosity of aqueous stool extract, and produce bulkier and more moist stools when compared to other fibre sources in corresponding amounts (Marlett et al., 2000). Researchers were able to extract fibrous gelatinous mass from the stool of participants who consumed psyllium, while the control group did not have the gelatinous fraction of the stool. It was concluded that a fraction of psyllium is not fermented (Marlett et al., 2000). In a randomized control trial studying faecal incontinence, it was reported that only psyllium (16 g of fibre per day for 32 days, compared with placebo, carboxymethylcellulose, or gum arabic) resulted in a visible gel in a stool (Bliss et al., 2014). The formation of a gel in faeces and the “firming consistency” of psyllium was proposed as a mechanism for decrease in faecal incontinence frequency. It was explained that the amount of psyllium is an important parameter - 16 g psyllium per day provided benefits which were not seen when participants took 7.1 g psyllium per day (Bliss et al., 2001). It was concluded that both the SCFA production as well as the unfermented fibre presence contributes to improvement of faecal incontinence (Bliss et al., 2001, 2014). In their review, McRorie et al. (2021) have discussed on the fermentability of psyllium and concluded that it is not fermentable and does not cause excess gas in *in vivo* studies. Moreover, it should be highlighted that *in vitro* studies are mostly done at low concentrations to

prevent gel formation and to allow for constant mixing, which could lead to fermentability, but does not reflect *in vivo* conditions when psyllium is consumed (McRorie et al., 2021; Slavin & Green, 2007). Interestingly, a recent study (Alhasani et al., 2025) showed that psyllium slowed down colonic transition of inulin, but did not change fermentation over 24 h period. Overall, it seems that fermentability could be influenced by the solubility of psyllium fibre, which will be discussed later in the section 5.2.

3. Development of *Plantago* mucilage extraction protocols and subsequent characterisation

3.1 *Plantago* mucilage structural properties

The extractability of fibres is largely determined by their chemical structure and properties. The majority of scientific literature regarding *Plantago* mucilage reports the use of varying solvents for polysaccharide extraction, while works using ultrasound, microwave, high pressure, and similar methods are sparse. The type of solvent affects the composition of the extract and, consequently, its characterization (Zhang et al., 2019). For example, if cold water is used for mixing the husk or the seeds, only the fibre fraction that is soluble at those conditions will be solubilised, while the rest will retain its gel formation. In psyllium, cold water extraction has shown to yield fibre with slightly negative charge and low gelling capacity (Yu et al., 2017; Zhou et al., 2020). On the other hand, the extraction with alkaline solvent will result in the higher fibre yield, as increased pH can disrupt the chemical bonds and network formation, swelling, and charge (Tejada-Ortigoza et al., 2016). In psyllium, alkaline extracted fibre showed high gelling capacity and strong viscoelasticity (Yu et al., 2017). In this section, an overview of *Plantago* mucilage extraction is presented, and proposed polysaccharide structure as a consequence of extraction steps are discussed. Table 1. shows an overview of publications in which fractionation protocol along with structure of *P. ovata* polysaccharide(s) were proposed.

The first *Plantago* species whose mucilage composition was researched was *P. psyllium* (Anderson & Fireman, 1935), though subsequent papers refer to the species

studied as *P. ovata*. This might reflect the common confusion between blond psyllium (*P. ovata*), the source of psyllium husk, and a much less common relative, dark psyllium (*P. psyllium* syn. *P. afra*) which does not possess a husk removeable by milling. Irrespective, it was reported that the mucilage consists of D-xylose, L-arabinose, and D-galacturonic acid, with its exact composition being dependent on the extraction conditions (temperature, seeds:H₂O ratio, time, and force applied to push the gel through the cloth) (Anderson & Fireman, 1935). Composition of aqueously extracted mucilage from *P. lanceolata* (Mullan & Percival, 1940) and *P. arenaria* (Hirst, 1951) were described as “similar”, containing xylose, arabinose and galacturonic acid, along with D-galactose which had not been reported before. The first reported multi-step extraction of *Plantago* mucilage consisted of extracting *P. ovata* husk in H₂O at 15°C, and then again at 90-95°C. The cold water extract had a higher uronic acid content and lower pentosan content than the hot water extract and the authors concluded that mucilage most likely consists of two distinct polysaccharides – one which is readily soluble in water and rich in uronic acids, and the other, which is neutral (Laidlaw & Percival, 1949; R. A. Laidlaw & Percival, 1950). Detailed structural analyses were required to determine how the constituent monosaccharides detected in these studies were assembled.

Kennedy and others (Kennedy et al., 1979; Sandhu et al., 1981) studied the structure of alkaline extracted *P. ovata* mucilage (1.2 M NaOH) using methylation analysis. They showed that the polysaccharide is highly branched (32.4% of all residues were branching points), but their proposed structure of a single polymer (xylan backbone with (1→3) and (1→4) linkages substituted at O-2 and O-3 with arabinose, xylose, and galactopyranosyluronic acid-rhamnose) was later proven to be incorrect. They were also the first to suggest that *P. ovata* gel most likely does not have covalent bonds. This was proven much later by Yu et al. (2017) and will be discussed later in detail.

One of the early papers that described mucilage characteristics for multiple *Plantago* species utilised one step acidic extraction with HCl (0.1 N) for eleven different types of *Plantago* seeds (Sharma & Koul, 1986). Importantly, the authors discovered that mucilage mass does not correspond to its water swelling properties – *P. ovata* had the highest mass of extracted mucilage while *P. psyllium* and *P. indica* had a higher

swelling factor (17.20 and 15.70, respectively, then followed by *P. ovata* whose swelling factor was 15.25 mL/g). This gave an early indication that even though *P. ovata* is the most widely used *Plantago* species, other cultivars have potential for food and nutritional purposes.

Based on monosaccharide composition and glycosidic linkage data from husk extracted with hot water (IHWE) and alkali (IHAE), Edwards et al. (2003) proposed an updated structure for arabinoxylans (AX) of *P. ovata* husk: the backbone is formed of β -D-(1 $\rightarrow$ 4)-linked xylopyranoses to which α -L-arabinofuranoses are attached via α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 2) linkages. The AX is highly branched with backbone substitutions (at a density that depended on extraction method) including α -D-galactopyranuronic acid (GalAp)-(1 $\rightarrow$ 2)-linked- α -L-rhamnopyranose-(1 $\rightarrow$ 4)- β -D-Xylp, α -D-GalAp-(1 $\rightarrow$ 3)-linked- α -L-Araf-(1 $\rightarrow$ 4)- β -D-Xylp, and α -L-Araf-(1 $\rightarrow$ 3)-linked- β -D-Xylp-(1 $\rightarrow$ 4)- β -D-Xylp. This was also the first study to report the structural differences and molecular weights of fractionated psyllium husk polysaccharides. IHWE had a molecular weight of 2200 kilodaltons (kDa) and only 18% of its xylose backbone residues were unsubstituted, while 31% were mono- and 11% were di-substituted, while IWAE has a molecular weight of 1600 kDa, and 20% of the xylose units were unsubstituted, 31% were mono- and 18% were di-substituted.

A similar structure for alkali-extracted *P. ovata* mucilage was published by Fischer et al. (2004): β -(1 $\rightarrow$ 4)-linked D-xylopyranosyl units forming the backbone, and single xylopyranosyl side chain residues at O-2, with trisaccharide L-arabinofuranosyl-(1 $\rightarrow$ 3)-D-xylopyranosyl-(1 $\rightarrow$ 3)-L-arabinofuranosyl side chains at position O-3. The authors developed a multi-step alkaline extraction process for *P. ovata* mucilage and reported the fractions to be neutral. Based on previously published results, some have reported the mucilage to be acidic while others found it to be neutral. It is sensible to assume that this discrepancy is a consequence of different extraction methods, and this was later confirmed by Guo et al. (2008). They reported structural data for three mucilage fractions (soluble in H₂O at 80°C (AE), soluble in 0.5 M NaOH (AES), and a gel fraction in 0.5 M NaOH (AEG)). AE and AES had up to 15% uronic acids, but AEG was neutral. The solubility and gelling properties were suggested to be explained by differences in sidechain motifs of the three fractions – branching of

the three fractions was 60%, 32%, and 74%, for AE, AES, and AEG, respectively. AE had the most complicated and longest side chains (up to 3 monosaccharide subunits), and AEG had short and regular side chains which could allow closer intermolecular interactions and explain its high gelling capability.

It is important to note that the xylans in *P. ovata* mucilage are different from cereal bran xylans, which are considered true arabinoxylans (AXs) (Kiszonas et al., 2013) because although their chemistry is similar, it is distinct. While both contain xylose backbones, cereal bran xylans are substituted solely with arabinose residues making them true arabinoxylans (AXs). On the other hand, *P. ovata* mucilage xylan sidechains are much longer and contain xylose. This is considered to preclude the *P. ovata* polysaccharide from being defined as a true arabinoxylan, as it is often labelled, and it should more correctly be classified as heteroxylan (HX). Secondly, most backbone residues of *P. ovata* HX are substituted, while unsubstituted xyloses are prevalent in cereal bran AX. Lastly, *P. ovata* mucilage HX shows decreasing water solubility with increasing arabinose to xylose (A:X) ratios, which is opposite to the behaviour of cereal bran AX. These findings point to a set of structurally diverse HX molecules comprising *P. ovata* mucilage, and likely applicable to the HX of other *Plantago* species, that confer highly unique functionalities.

Table 1. Overview of research papers where the structure of *P. ovata* polysaccharide was proposed, along with the corresponding extraction processes.

Reference	Extraction process	Experimental determination	Proposed structure
Kennedy et al., 1979 Sandhu et al., 1981	One step alkaline (1.2 M NaOH)	Gas-liquid chromatography and mass spectroscopy of methylated fractions Linkage analysis	Highly branched polymer (xylan backbone with (1→3) and (1→4) linkages substituted at O-2 and O-3 with arabinose, xylose, and galactopyranosyluronic acid-rhamnose)
Edwards et al., 2003	Extraction in H ₂ O Extraction in alkali (0.1 M NaOH)	High performance anion exchange chromatography with pulsed amperometric	Linear β-D-(1→4)-linked xylopyranose backbone with side residues: α-L-arabinofuranose units are attached via α-(1→3) and α-(1→2) linkages, α-D-glucuronopyranose (GalAp)-

	Other conditions not specified	detection (HPAEC-PAD) Methylation analysis for uronic acids	(1→2)-linked- α -L-rhamnopyranose-(1→4)- β -D-Xylp-(1→4), α -D-GalAp-(1→3)-linked- α -L-Araf-(1→4)- β -D-Xylp, and α -L-Araf-(1→3)-linked- β -D-Xylp-(1→4)- β -D-Xylp
Fischer et al., 2004	3 fractions were extracted: A – alkali insoluble, B – alkali insoluble and acid soluble, C – alkali and acid insoluble (protocol by Marlett & Fischer, 2001)	Gas-liquid chromatography and mass spectroscopy of aldol-acetate derivatives NMR spectroscopy	β -(1→4)-linked D-xylopyranosyl units forming the backbone, and single xylopyranosyl side chain residues at O-2, with trisaccharide L-arabinofuranosyl-(1→3)-D-xylopyranosyl-(1→3)-L-arabinofuranosyl side chains at position O-3
Guo et al., 2008	Extraction in H ₂ O at 80 °C Alkaline extraction 0.5 M NaOH Alkaline extraction 4. 1.2 M NaOH Alkaline extraction 2 M NaOH (all alkaline extractions - room temperature, 2 h)	HPAEC-PAD for monosaccharides GC-MS for methylated fractions Dynamic light scattering for hydrodynamic radii determination	Fraction 1: 1→4) and 1→3) linked β -D-xylopyranosyl residues in the main chain with terminal arabinose and xylose connected to the main chain by O-3 and/ or O-2 linkage in side chains Fraction 2: 1→4 linked β -D-xylopyranosyl residues in the backbone and side chain consisting of arabinosyl residues linked through O-3 position

3.2 *Plantago* mucilage functional properties

As newfound knowledge of the chemistry of whole and fractionated *P. ovata* mucilage polysaccharides was acquired, the interest increased in studying their secondary structure and intermolecular interactions that result in their desirable viscoelastic properties. More detailed information could unlock the drivers behind its unique gelling behaviour and guide their use in providing beneficial health properties and applicability in food, pharmaceutical, and engineering industries. Therefore, attention was rapidly focused on the rheological properties of *P. ovata* mucilage.

In one of the earliest studies of its rheology, (Haque et al., 1993) *P. ovata* mucilage was compared to the xanthan gum. Alkali extracted (2.5 M NaOH, room temperature) *P. ovata* husk mucilage was described as weak gel whose structure is broken down under steady shear rotation, but which is retained under small deformation oscillatory measurements. In addition, melting of the gel was described as a continuous process happening above 80°C.

Since Haque et al.'s pioneering study, Farahnaky et al. (2010) have employed amplitude (strain) sweep, frequency sweep, and temperature sweep tests under varying conditions (temperature and pH) to assess the rheology of *P. ovata* mucilage, which was presumably extracted at 25 °C, although the method section is lacking in detail. As expected, an increase in the gel concentration from 2 to 2.5 and 3% resulted in moduli increase due to the higher viscosity, and interactions and entanglements between the polymer chains. G' , which is the measure of deformation energy stored in the sample during the shear measurement, i.e. the elastic behaviour of a sample, was higher than G'' - the measure of deformation energy lost in the sample during the shear process, i.e. the viscous behaviour of a sample, at all tested concentrations. Frequency sweep tests showed G' and G'' curves almost parallel to each other, thus displaying characteristics of a weak gel, as opposed to entanglement systems, which display an intersection of G' and G'' . Moreover, $\tan \delta$ (the ratio between elastic and viscous behaviour i.e. $G':G''$) for all concentrations was lower than 0.2 indicating a weak gel structure since $\tan \delta > 1$ reflects a more liquid-like material while $\tan \delta < 1$ reflects a more solid-like material. Temperature sweeps showed a long and continuous melting process where G' and G'' of the 2.5% gel showed a plateau at temperatures above 40°C. Frequency sweep tests of a 2.5% gel at different pH values showed decreased G' and G'' , but higher $\tan \delta$ at low pH (2.5), indicating that the gel has lost its elasticity due to lower electrostatic repulsion at lower pHs.

A series of papers published by Yu et al. (2017, 2018, 2019) presented in-depth structural and rheological characterisation of *P. ovata* mucilage, as well as proposed mechanisms that govern the unique behaviour of different mucilage fractions. Three fractions were isolated, namely a cold water (CW) fraction extracted with H₂O at 25 °C, a hot water (HW) fraction extracted with H₂O at 65 °C, and a KOH fraction

extracted under alkaline conditions with 0.2 M KOH. Their monosaccharide composition agreed with previously published results – the CW fraction had the highest amount of acidic sugars and rhamnose, while the arabinose to xylose ratio increased along with increasingly intense extraction steps, giving A:X ratios of 0.20, 0.30, and 0.33 for three consecutive fractions. HW and KOH fractions had strikingly similar monosaccharide composition, linkage analysis patterns, and molecular weight (HW – 971 kDa, KOH – 953 kDa), however their rheological characteristics were very different. The CW fraction can be described as a viscoelastic fluid ($G'' > G'$ or $\tan \delta > 1$). While HW and KOH fractions both display gel-like behaviour, KOH is frequency-independent (thus, can be classified as a true gel) while HW is frequency-dependent, (meaning that it is more liquid-like than KOH). Moreover, KOH gels retain their mechanical properties up to 85°C, but melting of the HW fraction begins at 41 ± 2 °C and HW shows a gradual decrease in G' as the temperature increases. The authors have proposed that both gels are physical, meaning that hydrogen bonding is responsible for the gel-like properties, as opposed to covalent bonding. To show this, experiments using 8 M GuHCl (a strong chaotropic agent that disrupts hydrogen bonding) proved to have significant effects on the rheological profile and it dissolved gels from both fractions. Since ferulic acid residues are a primary driver for covalent bonding in arabinoxylan gels (as shown for cereal arabinoxylans (Kiszonas et al., 2013), and *P. ovata* HX have very low amounts of ferulic acid residues (Yu et al., 2017), the proposed reason for different behaviours of HW and KOH fractions is attributed to differences in their side chain distribution and hydrogen bonding of side chain to another side chain or to the backbone, which then drives its rheological behaviour (Yu et al., 2018). Moreover, it was shown that differences in temperature or solvents used have an impact on the intricate structure and polysaccharide motifs, and removal of terminal arabinoses in sidechains dramatically influences rheological behaviour, thus confirming that hydrogen bonding of side chains is the main force driving the gelling behaviour of these mucilage fractions (Yu et al., 2019).

In a study by Ren et al. (2020a), five different fractions of *P. ovata* husk mucilage were aqueously extracted under increasing temperatures (20, 40, 60, 80, and 100 °C). It was reported that with temperature increase, arabinose to xylose (A:X) ratios

increased as well (0.298, 0.305, 0.322, 0.363, and 1.979, respectively), indicating very different polymers isolated that would have correspondingly different functional properties. F20 (a fraction extracted at 20 °C) was the only fraction that was influenced by thermal treatment – its mechanical spectrum showed differences before and after heating, while mechanical spectra of other fractions overlapped. The authors have proposed that F20 might be substituted with single xylose units and simple side chains which can partially associate and form a weak gel at low temperature, but are influenced by higher temperature and, therefore, show differences before and after first and second heating. On the other hand, fractions extracted at higher temperatures had more complicated side chain motifs that restrict associations at higher temperature and are probably supported by hydrogen bonds, thus forming a ‘physical’ gel which retains its rheological properties even after first and second heating.

Similarly, Zhou et al. (2020) have characterised two fractions from *P. ovata* husk mucilage. A cold extracted fraction (CP, 25 °C) had five times more uronic acids than a hot extracted fraction (HP, 85 °C) at 18.5% and 3.5%, respectively. The molecular weight of CP was 630 kDa, while HP was 2100 kDa. CP exhibited behaviour consistent with a viscoelastic fluid, while HP displayed weak gel characteristics and had a critical gelation point of 4.8 mg/mL. Temperature sweep tests showed thermo-reversible properties of both fractions but melting temperature and gelation temperature showed a dependence on concentration. This supports the previously proposed theory of Yu et al. (2018) that weak gel behaviour is dictated by hydrogen bonds and polymer entanglements. Thus, it is possible that below gelation temperature, the gel-like behaviour of HP was facilitated by hydrogen bonds, but above gelation temperature, the polymers exhibit more movement, which contributes to viscous behaviour. Furthermore, steady flow sweep of CP showed shear-thinning behaviour and increased apparent viscosity with an increase in concentration, which can be explained by more random chain entanglements at higher concentrations which are then susceptible to shear.

With evidence that hydrogen bonds play a key role in gelling behaviour of *P. ovata* mucilage, it was then shown that these bonds can be described as “weak” or “strong”

in AX-W (arabinoxylan extracted with H₂O at 65 °C after extraction at 25 °C) and in AX-A (arabinoxylan extracted with KOH), respectively (Yu et al., 2021). By utilising a wide range of oscillatory measurements, it was possible to measure and calculate molar energy of hydrogen bond junctions for HWF (97–144 kJ/mol) and AEF (402–480 kJ/mol). The authors called the weak hydrogen bonds, which can be used to describe the rheological behaviour of HWF, as “fast self-healing”, while strong hydrogen bonds are “slow self-healing” and characteristic of AEF. Practically, this means that gelling behaviour is indeed driven by *P. ovata* polysaccharide motifs, as hydrogen bonds are primarily created between terminal xyloses and arabinoses in sidechains, and these differences could and should be exploited in fabricating different food products or packaging materials and for medicinal and pharmaceutical purposes.

While in the published literature, aqueous and alkaline extraction of *P. ovata* are the most common, employed either as one-step or sequential extraction processes, in recent years other types of extractions have been developed including those that do not require drying and reconstitution. For example, in a recent study Kaczmarczyk et al. (2023) hydrated husk in H₂O at 25 °C before centrifuging to produce a mucilage-rich supernatant they refer to as ‘raw extracts’. For research purposes reconstituted extracts are used, meaning mucilage is extracted, dried, and then rehydrated to get the exact concentration for further experiments. In this case, the polysaccharide concentration in raw extracts was not presented so polysaccharide concentration was not controlled. As concentration is a factor critically influencing rheological properties, the results cannot be interpreted as 1-for-1 comparisons of functionality between fractionated polysaccharides but might be insightful to end-users who wish to perform a simple fractionation to get targeted properties. As such, the study of raw extracts corresponds to industry or household use of psyllium husk. To this end, the authors show that the raw extracts can be described as viscoelastic fluids with two types of polymer chains whose molecular weights are 200 and 1780 kDa.

Mucilage properties can be affected by different conditions before and after extraction, and even before seed harvest. Increased rainfall at *P. ovata* crop maturity leads to excessive moisture at the hydrophilic seed surface which results in changes

in seed and husk colour and diminishes the water absorption capacity, which is a benchmark of psyllium utility (Cowley et al., 2022). Crops that were subjected to unseasonable rain had lost part of the most soluble outer layers of mucilage along with increased microbial infestation and poorer germination. Even though rheological properties of mucilage were not tested, its quantity and quality were impacted, thus, it is reasonable to assume that such exposure would affect the functional properties as well. Therefore, when impacted by uncontrollable (such as rain) and tractable (such as drying) processes, it is important to acknowledge that raw extracts (rather than extracts that are dried and reconstituted at known concentrations) do not offer the same scientific precision as reconstituted extracts, especially as it is known that rheological properties are affected by concentration of polysaccharides.

As it has become clear that the gel properties are influenced by extraction methods, such methods specifically tailored for research purposes and for application in the food and health sectors have begun to be developed. Cowley et al. (2020) streamlined a small-scale fractionation process which can be used to extract three different fractions of mucilage by shaking the seeds in H₂O at 25 °C, then at 65 °C, and finally, seeds were subjected to intense agitation using a tissue disruptor-type mixer mill. The entire process takes less than 5 h, and offers an alternative to alkaline extraction, which can be tedious to neutralise and dialyse. The protocol is applicable not only to *Plantago* seeds but also for other mucilage producing seeds when screening myxospermous germplasm to assess seed quality and for chromatographic analyses.

Overall, various extraction processes and fibre characterization have been reported for psyllium. Due to its viscous nature, extracting the gel in its entirety required multi-step extraction. Consequently, fibre fractions were reported to be different in structure and in rheological profile. As shown, there are many research papers describing the structure of said fractions. However, the application of these fractions is still underexplored. It is sensible to assume that fibre fractions with different rheology properties would have different structuring potential in food, which should be further explored. Therefore, the next chapter brings an overview of challenges

when applying psyllium in food products and, further on, solutions are presented based on the variability of extracted psyllium fractions.

4. Positive and negative impacts of psyllium fibre implementation

Research has shown that most people do not meet the recommended dietary fibre intake: for example, Europeans eat around 78% and Americans 62% of the recommended intake (McKeown et al., 2022). Fibre can be ingested in two ways: in an isolated form as a supplement or in a food product, either added or naturally present. Oat bran or psyllium husk are common examples of fibre supplements, which people usually take with water (as recommended). This is the most common way to assess health effects of a fibre in clinical studies, however, this is most likely not representative of typical diets consumed by people, unless they have been advised by a health practitioner. Eating whole foods rich in fibre is typically the first advice when recommending increasing fibre intake (McKeown et al., 2022), which is why the food industry frequently aims to enrich food products. Additionally, it has been shown that health claims have an impact on consumers' choices (Benson et al., 2019). According to EFSA, a food product can have specifically worded claims related to reduction of glycaemic response and healthy gastrointestinal function if it supplies 3.5–14 g of psyllium, inulin, oat bran, or flax groats (EFSA, 2010) in its daily serving. If a food product contains 1–3 g of psyllium seed husk per its daily serving, it can have health claims related to satiety (EFSA, 2010). However, adding fibre to a food product can cause negative organoleptic changes (Foschia et al., 2013). Using baked goods as a commonly fibre-fortified example, insoluble fibre can cause lower bread volume or darker crust colour, while soluble fibre can change dough viscosity and ultimately bread texture. As detailed in the introduction, *Plantago* mucilage has many applications in food and health industries. Previously described mechanisms (Section 2) behind its unique gelling behaviour are also related to its medicinal benefits (illustrated in Fig. 1), but also make it difficult to include adequate amounts of fibre in diet and food products. In this section, the challenges of *P. ovata* husk and mucilage implementation in food are highlighted, followed by the examples of how its unique properties can be both advantageous and adverse (Fig. 2).

Psyllium is an important structuring agent in gluten-free breadmaking, but its incorporation requires increased water addition to maintain quality. However, too much water can lead to bake loss, decreased volume and lower bread acceptability. Fratelli et al. (2018) modelled how the addition of various amounts of psyllium (2.86–17.14%) and water (82.14–117.86%) in gluten free (GF) bread affects organoleptic qualities. The authors demonstrated that the addition of psyllium up to 10% is possible without altering water addition to achieve acceptable bread with improved textural properties. Nevertheless, bread with 2.86% psyllium and 82.14% water addition had highest overall acceptability (8.72 on a 10-point scale). Bread with 17.14% psyllium and 117.86% water had decreased glycaemic index *in vivo* (-25%), but lower acceptability with a score of 7.11. This work demonstrates the discrepancy between psyllium addition levels that yield the best acceptability scores, and the psyllium addition levels necessary to improve glycaemic response. When psyllium and HPMC are added to GF rice bread, it was found that psyllium decreased bread volume and increased hardness, proportional to its addition (0–4 g/100 g), but higher water addition (90–110 g per 100 g of flour) mitigated the negative traits to a certain extent (Mancebo et al., 2015). The usefulness of psyllium addition in non-model GF doughs (corn starch, amaranth flour, pea isolate) was confirmed by Mariotti et al. (2009): 2% psyllium provided a cohesive network to support the starchy matrix and created a rheologically stable system with a longer linear viscoelastic region. It is important to mention that the authors varied water addition, as per farinograph measurements, to ensure workable GF dough and thus water deficit was at least partly accounted for. The difference between the quantity of psyllium which would provide desirable products and the amount which would have significant health benefits was also pointed out in a recent review by Belorio & Gómez (2022). Even so, psyllium continues to be important in the GF bakery industry but Roman et al. (2019) have highlighted the gap between commercial use and research of psyllium fibre in GF bread. They noted that incorporation of pectin is widely studied, even when it is not found in commercial GF breads, whereas psyllium should be researched more, considering that it is commercially widespread.

Psyllium has also been applied to gluten-containing products. Unlike in gluten-free breads where psyllium is a structuring agent, psyllium is added to gluten-containing products typically to improve nutrition. However, in these systems, there is often competition for water between psyllium and gluten, and optimisation is needed. To determine the optimal amount of psyllium addition and hydration in wheat bread, Mironeasa and Codină (2023) have used response surface methodology to model how four levels of psyllium addition (0, 2, 4, 6%) and hydration (60, 65, 70, 75%) impact bread physical properties (retention coefficient, loaf volume, porosity, and elasticity). It was concluded that bread with 3.53% psyllium and 75% hydration would yield the best physical properties. Even though it was possible to find an optimum recipe for psyllium-enriched bread, all rheology properties during dough development (maximum height of gaseous production, total gas volume, gas retained in the dough) were negatively impacted when psyllium was added i.e. 0% additions were superior. In another study, a 10% wheat flour replacement with psyllium increased slowly digestible starch and decreased rapidly digested starch when compared to control wheat bread, but psyllium enriched bread had lower volume and porosity, and increased hardness and chewiness (Bilgic & Sensoy, 2023). Additionally, psyllium decreased hardness in wheat flour crackers which is considered a negative effect as hardness in crackers could be related to fracturability and crispiness, which is desirable in crackers. One possible reason for these effects could be the fact that water addition was not adjusted when psyllium was incorporated. Raymundo et al. (2014) found that psyllium can be incorporated into wheat biscuits from 3 to 9%, but higher psyllium replacement (up to 15 %) resulted in unworkable dough which could not be shaped. Psyllium (3 – 9 %) caused higher spread ratios, increased hardness, and lower cohesiveness, indicating that dough with psyllium affected internal cohesion leading to altered texture. In a continuation of this work, Fradinho et al. (2015) found a similar trend: increasing psyllium in biscuit formulation negatively affected texture and colour. The overall sensory acceptance was highest in biscuits with 3.9% psyllium and 51.4% wheat flour, but the optimal formulation determined by response surface methodology was 6% psyllium and 48% wheat flour.

It is important to note that as bakery products are typically made from starch-rich flours, they can cause blood glucose spikes which are a risk factor for metabolic syndrome. Some fibres are well-known to reduce the glycaemic index of starchy foods but there are many mechanisms, and this has not been well-studied for *Plantago*. In one rare study, it was shown that psyllium addition to wheat, potato, or tapioca starch can reduce starch gelatinization (Sevilimis & Sensoy, 2022), however this effect is seen when psyllium addition is very high (psyllium:starch=1:1). A 50% addition to food is an unattainable and potentially hazardous quantity of psyllium to be implemented in an actual food product. However more realistic (but still high) additions (17.14%) have seen large reductions in glycaemic index and glycaemic load *in vivo* (Fratelli et al., 2018). The underlying mechanism is not known and the influence of psyllium on starch digestibility properties, such as gelatinisation degree, granule swelling, rupturing, and structure after thermal treatment should be further explored.

Beyond baked goods, psyllium can be added into fermented dairy products as a fat replacer and to decrease syneresis (release of water from the food product during storage). When psyllium was incorporated into yoghurt at levels of 0.1, 0.3, 0.5, and 0.7%, it was found that the highest addition (0.7%) resulted in the highest microbial count and ash content (Bhat et al., 2018). Flavour was negatively impacted by psyllium incorporation however, along with textural attributes (hardness, gumminess, and adhesiveness). However, yoghurt with 0.5% had the highest acceptance.

When developing pectin-based gels that can be used as a model for fruit jams and declared as a “source of fibre”, psyllium-enriched gels (3 g per 100 g of gel) showed no syneresis and had high cohesiveness, but had excessive gumminess, low fracturability and hardness (Figueroa et al., 2018). However, when in combination with other fibres (bamboo, apple, or wheat) psyllium gels showed desirable texture and stability. In a further study focussed on apple jam (Figueroa & Genovese, 2019), the authors again confirmed increased gumminess of the jam when psyllium was used alone. In combination with other fibres, it resulted in improved texture and mouthfeel, as well as stability up to 30 days.

In a study by Ferjančič et al. (2021), low fat chicken sausages were prepared with 3 % or 6 % psyllium husk addition to replace the missing fat component. As expected, enriched sausages had higher fibre content, though psyllium did not negatively affect texture properties. However, colour was significantly changed. In sensory evaluation, sausages with 3% psyllium addition were accepted by consumers, but sausages with 6% addition received lower scores, particularly regarding mouthfeel, juiciness, and appearance.

Overall, psyllium has a positive influence on a variety of food products – it is a common improver of GF bread, and it could be implemented in meat replacement products, especially as their popularity is rising. However, psyllium can cause lower product quality when implemented in sufficient quantities to meet a “high in fibre” claim, which is 6 g of fibre per 100 g of product, or 3 g of fibre per 100 kcal ((European parliament, 2006)). Its high water absorption capacity and rheological properties represent certain technological challenges, which makes it unsuitable for all applications. Oftentimes positive effects are closely linked with certain negative traits. In the next section, the possible avenues for expanding *Plantago* mucilage use through natural or induced variation in chemical and physical properties are presented.

Positive impacts of psyllium addition	Aim of psyllium addition	Negative impacts of psyllium addition
Improves bread texture and volume, can reduce glycaemic response	To improve the dietary fibre content and glycaemic response of GF Bread Fratelli <i>et al.</i> , 2018	Lower acceptability score due to excess water impacting crumb and crust attributes
Can improve health properties, and technological properties of GF bread	To develop GF bread with higher protein and fibre content Mariotti <i>et al.</i> , 2009	Requires formulation optimization (specifically water content), which can increase cost and time
When dough hydration was optimised (75%), breads containing psyllium (3.53%) had improved volume, porosity and elasticity	To produce fibre-fortified wheat breads with optimal fermentation and physical characteristics Mironeasa & Codină, 2023	Dough stability during fermentation was decreased from psyllium interrupting starch-gluten networks
Decreased rapidly digested starch, and increased slowly digested starch	To restrict starch gelatinisation and digestibility Sevilimis & Sensoy, 2022	Psyllium addition needs to be high to show the effect of restricted gelatinisation, which is not practical in food product development
Higher fibre and mineral content	To produce healthier biscuits with higher fibre content Raymundo <i>et al.</i> , 2014	Increased spread ratio and hardness, decreased cohesiveness
Higher incorporation (0.7%) resulted in proportionally higher microbial count	To fortify yoghurt with fibre Bhat <i>et al.</i> , 2018	Lower incorporation (0.5%) had better overall acceptance Higher psyllium incorporation caused increased hardness and gumminess
All psyllium enriched gels showed no syneresis	To achieve 'source of fibre' claims in fruit jams Figuroa & Genovese, 2018; 2019	Psyllium caused undesirable gummy attributes
Psyllium increased fibre content and did not change texture properties	To replace fat in low fat chicken sausages Ferjancic <i>et al.</i> , 2021	Psyllium addition caused darker colour, and had lower sensory acceptance

Figure 2. The variety of food uses of psyllium husk and their positive and negative impacts on product quality.

5. Strategies to broaden *Plantago* mucilage functionality and implementation

Altogether, there is rich literature highlighting the unique and commercially important properties of *P. ovata*-derived psyllium husk and its mucilage. However, there is evidence in the literature that would suggest that there is little genetic variation among cultivated *Plantago ovata* accessions (Dhar et al., 2005; Kour et al., 2016) and therefore likely very little variation in functionality that can be exploited for targeted industrial use. Certainly, the many studies of *P. ovata* mucilage chemistry and rheology from researchers around the world yield remarkably similar results (covered in detail in Section 3). It is possible that psyllium's functionality could be improved or made more potent, reducing demand and tempering the fluctuations in price. It may further improve its properties as a dietary fibre. Fig. 3 shows five common limitations of psyllium husk implementation in food products, and the possible solutions that have been studied and are shown to be successful, with brief description of the mechanisms through which those improvements are achieved.

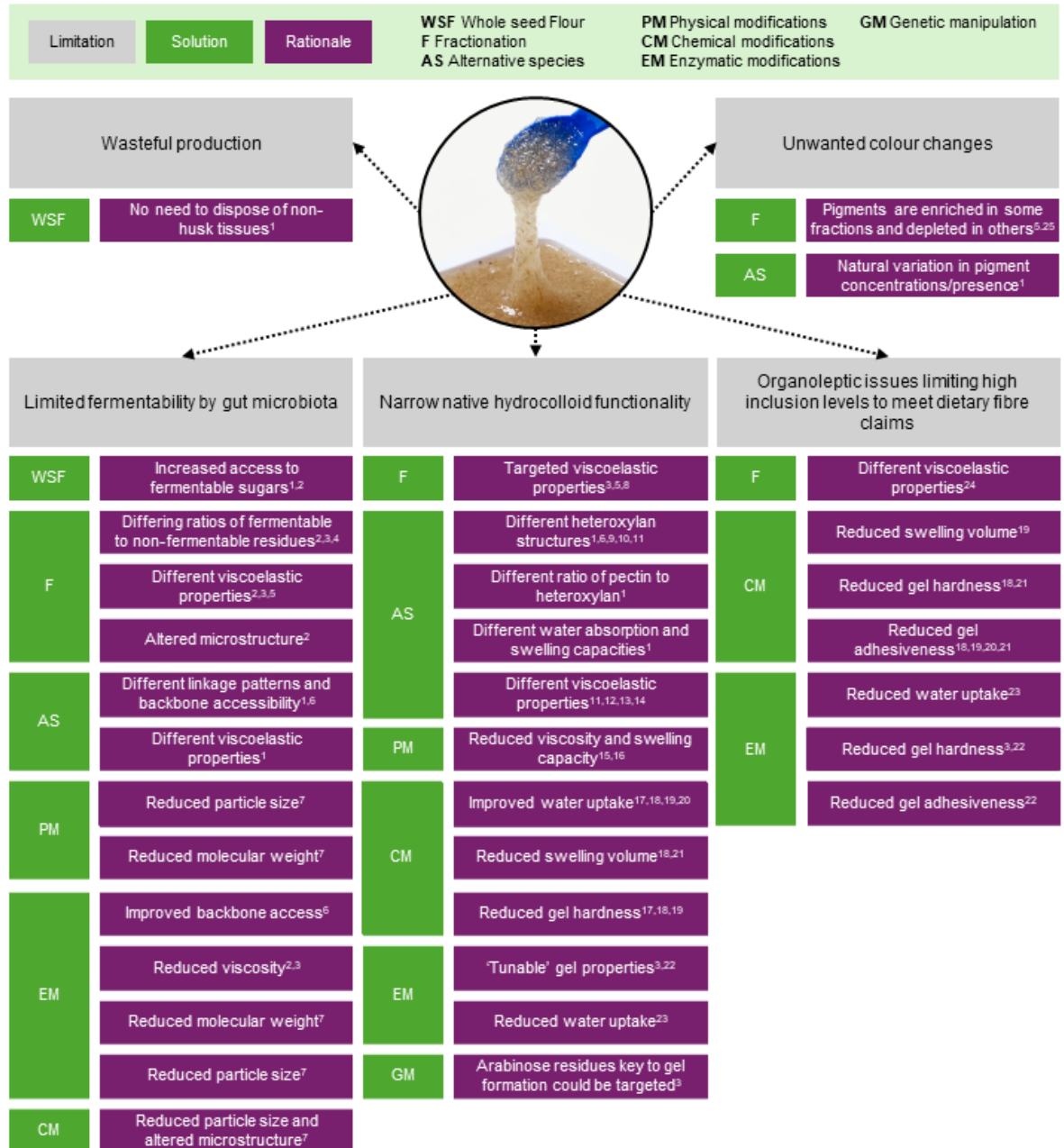


Figure 3. An overview of key limitations associated with implementation of psyllium husk in products. Strategies from Section 3 that may have promise as solutions to overcome these limitations are included along with a high-level rationale.

Specific precedents associated with the proposed rationale are included as superscript numbers: (1) Cowley et al., 2021; (2) Harris et al., 2023; (3) Marlett & Fischer, 2003; (4) Yu et al., 2017; (5) Ren, Yakubov, et al., 2020; (6) Phan et al., 2016; (7) Pollet et al., 2012; (8) Zhou et al., 2022; (9) Zhao et al., 2014; (10) Addoun et al., 2020; (11) Benaoun et al., 2017; (12) Yin et al., 2016; (13) Hesarinejad et al., 2018;

(14) Behbahani et al., 2017; (15) Mallikarjunan et al., 2024; (16) Mallikarjunan et al., 2021; (17) Liu, Zhang, et al., 2010; (18) Niu et al., 2012; (19) Niu et al., 2013; (20) Cheng et al., 2009; (21) Liu, Xie, et al., 2010; (22) L. Yu et al., 2003; (23) L. L. Yu et al., 2008; (24) Fradinho et al., 2020; (25) Niu et al., 2019.

5.1 Use of other *Plantago* species

P. ovata is the only *Plantago* species utilised commercially for its seed and fibre, even though *at least* 77 of them produce mucilage (Cowley & Burton, 2021). One reason for this is related to ease of processing and the anatomical nature of how the mucilage polysaccharides are stored in the mature seed: psyllium husk is a cell-less layer of dried, laminated polysaccharides that can easily be milled from the seed surface. To our knowledge, only *P. ovata* (and perhaps closely aligned relatives) can be processed in this way as other species have mucilage stored in discrete cells that are not easily separated from the seed (Cowley & Burton, 2021). For this reason, *P. ovata* is the most extensively studied *Plantago* species despite underutilised relatives showing promise and recently becoming more studied (Fig. 4). In fact, mucilage from non-*P. ovata Plantago* species can be applied to products through extraction methods similar to those used for psyllium husk or the use of whole seeds and whole seed flour. Thus the potential for exploiting natural variation in *Plantago* mucilage properties for tailored end uses is substantial.

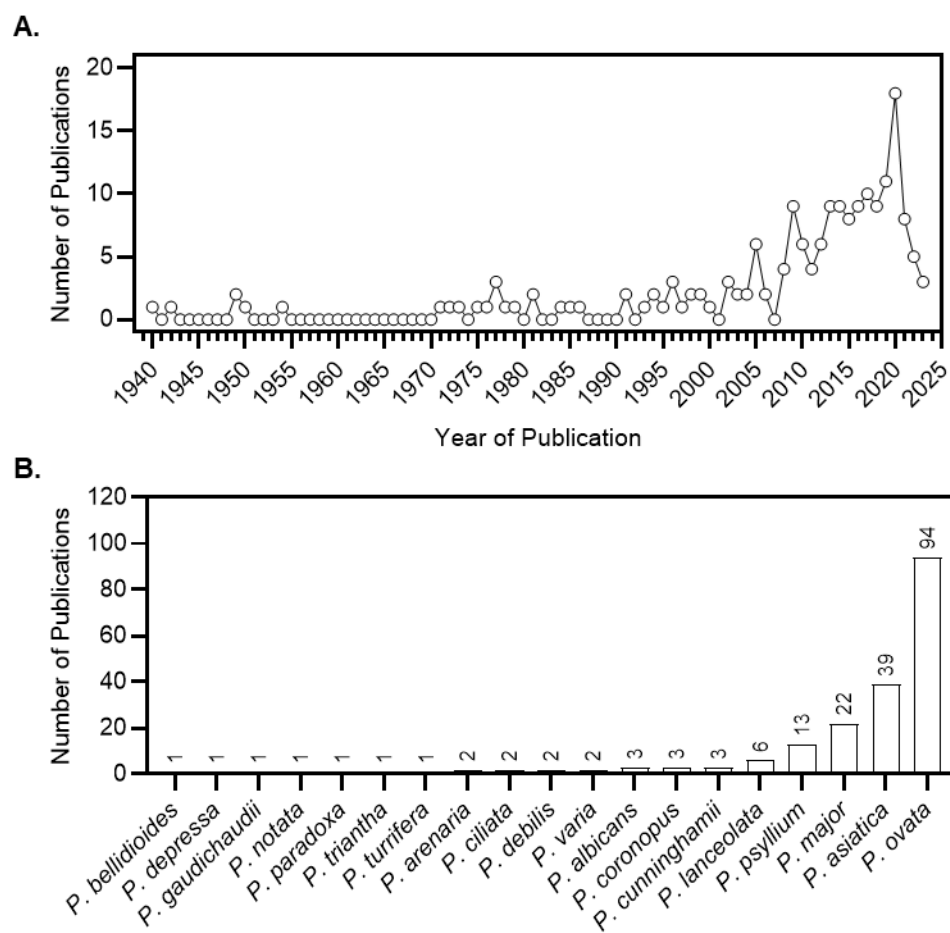


Figure 4. Bibliometric data of publications relating to *Plantago* seed polysaccharides between 1940 and 2023 (A) then delineated by species studied (B).

The search engine Scopus was used with the term “TITLE-ABS-KEY (plantago AND seed) AND polysaccharide OR mucilage OR hydrocolloid OR gum AND NOT (musa). “*Musa*” was excluded to save screening and excluding large numbers of articles on starchy banana as the common name “plantain” is shared by both *Musa* and *Plantago* species and often results are shared. Entries ($n = 312$) were manually screened to remove non-relevant articles and the species studied in articles deemed relevant ($n = 19$) were identified and the number of publications in which they were each studied recorded.

In a comparative study of twelve *Plantago* species native to or naturalised in Australia, mucilage traits varied significantly between the studied species (Cowley et al., 2021). Mucilage yield ranged from 23.84% (*P. ovata*) to 4.43% (*P. paradoxa*), though most species were higher yielding (median = 15.52%, per seed weight). Water absorption capacity was also tested showing similarly wide variation: *P. turrifera* had the highest water absorption capacity (22.20 mg H₂O/mg seed), and *P. triantha* the lowest (1.42 mg H₂O/mg seed). Interestingly, the relationship between yield and water absorption capacity is not perfectly linear. This is consistent with some earlier studies comparing *P. ovata* to its wild allies (Sharma & Koul, 1986), suggesting that the chemical differences detected result in particular mucilage functionalities not explained entirely by polysaccharide concentration. Several studies have delved deeper into linking chemistry with functionality for mucilage polysaccharides from non-*P. ovata* *Plantago* species.

P. depressa mucilage (extracted at 100 °C) was divided into four fractions using chromatography (Zhao et al., 2014). All four fractions had high molecular mass (> 1200 kDa) and were protein-bound. Their composition included acidic polysaccharides with relatively high arabinose (up to 55.7%) and fucose (up to 28.7%) content, but low xylose (up to 11.6%), which is quite different to *P. ovata*. Fraction 3 had a distinctly high mannose content (28.1%). All fractions, and in particular Fraction 3, showed immunomodulatory effects, measured as lymphocyte proliferation, TNF- α (tumour necrosis factor α) production and NO (nitric oxide) content (Zhao et al., 2014).

P. asiatica alkaline-extracted mucilage (water extractable fraction was discarded prior to alkaline extraction) was identified as a highly branched arabinoxylan with 20.5% uronic acid and an A:X ratio of 1:4.1 (Yin et al., 2016). Rheologically, the mucilage displayed shear thinning behaviour, depending on concentration and frequency, as well as thermal hysteresis ($G' = G''$) at 55 °C. Based on current knowledge, thermal hysteresis is unique to this *Plantago* species. Interestingly, the alkaline fraction from *P. asiatica* was characterised as a weak gel, unlike the alkaline extracted fraction from *P. ovata*, which is described as a true gel. When Ca²⁺ and Na⁺ were added, G' and crossover temperature were increased. Moreover, a Ca²⁺ gel

showed lower frequency dependence, higher G' , and superimposed heating and cooling curves. Therefore, its behaviour was more representative of a true gel.

Behbahani et al. (2017) have used response surface methodology to determine the optimal conditions for the aqueous extraction of mucilage from *P. major*, which were: 75 °C, pH 6.8, with a seed to water ratio of 1:60. These conditions yielded 15.18% extracted polysaccharide whose molecular weight was 1200 kDa, that displayed high total phenolic and flavonoid contents (76.79 mg GAE/g and 97.8 mg/g, respectively) and could be used as powerful antioxidants in food products. The extract showed applicability as an emulsification agent and foam stabiliser (67.4% emulsion stability and 88.4% foam stability).

P. ciliata polysaccharides were aqueously extracted (at 60 °C) at a yield of 18.6% (Addoun et al., 2020). They were characterised as heteroxylans with an A:X ratio of 1:4, and molecular weight of 700 kDa. The rheological properties were described as a weak gel, both in water and salt solutions. When considering health properties, *P. ciliata* showed anti-hyperglycaemic and anti-inflammatory effects (Addoun et al., 2021). The extract inhibited activity of α -amylase, presumably due to fibre enveloping starch granules which then blocks the access of α -amylase. Moreover, it showed inhibition of α -amylglucosidase, possibly due to its highly branched structure.

A water extractable (60 °C) fraction of *P. notata* was characterized as heteroxylan with a predominant composition of xylose, rhamnose and arabinose (77.4, 9.20, 7.58 mol%, respectively) and a molecular weight of 2300 kDa (Benaoun et al., 2017). Rheologically, the fraction can be characterised as a weak gel. Interestingly, the authors have described the xylan backbone as having a combination of both β -(1,4) and β -(1,3) linkages. While a mixed-linkage xylan from *Plantago* has been proposed before, the current scientific consensus is that β -(1,3) linkages exist in unusual sidechains rather than the backbone (Cowley & Burton, 2021).

P. lanceolata seed mucilage solution was characterised as weak gel with slight frequency dependence (Hesarinejad et al., 2018). When increasing the concentration (0.5 to 2 %), complex viscosity increased, which points to a usefulness of *P. lanceolata*

mucilage as a food thickener or stabiliser. Heating and cooling cycles showed hysteresis, but it decreased with lower heating-cooling rates, pointing to formation of hydrogen bonds and a gel network. In this work (Hesarinejad et al., 2018), polysaccharides from *P. lanceolata* were aqueously extracted, presumably at room temperature for 1h (temperature was not specified). Monosaccharide composition primarily consisted of mannose (69.42 %), arabinose (11.98 %), glucuronic acid (7.45 %) and xylose (7.42 %), and as such the polysaccharide is described as a 'arabinoxylomannan'. However, when compared to other published results (Cowley et al., 2021, and new data still not published), xylose is the dominant monosaccharide in *P. lanceolata* gel. A possible explanation could be mannose leaching from endosperm during the extraction process which has been observed previously (Cowley et al., 2020), but considering that the extraction was not long (1 h) or harsh (room temperature), this is most likely not the case. A likely explanation could be monosaccharide analysis itself – HPAEC-PAD was used to determine sugar composition, and the overlap of xylose and mannose is a widely known problem in this system (Nagel et al., 2014). It is likely that the reported high amount of mannose is actually xylose. *P. lanceolata* gel composition should be confirmed, but rheological data still suggest its application in food systems is very much a viable option.

5.2. Use of fractionated *Plantago* mucilage

As shown clearly for *P. ovata*, mucilage fraction chemistry and functionality are influenced by extraction protocols (Section 2). Studies that have prepared specific fractions of *Plantago* mucilage using methods like temperature fractionation are discussed. Therefore, using non-modified specific fractions (instead of whole husk) could be a useful option to overcome the challenges presented in the previous section.

In a study of twelve diverse *Plantago* species (Cowley et al., 2021), mucilage was fractionated by a simple method employing temperature and agitation (CWE = cold water extract, HWE = hot water extract, IAE = intense agitation extract) (Cowley et al., 2020). The highest pectin to heteroxylan ratio was found in CWE of *P. bellidioides*, followed by *P. coronopus* (1.99:1 and 1.40:1, respectively). On the other hand, *P. major* and *P. lanceolata* showed low pectin components (0.23:1 and 0.34:1,

respectively). A:X ratio is an important factor in assessing arabinoxylan properties, as it indicates degree of branching, which relates to extractability and susceptibility to enzymatic hydrolysis. The trend shows an increase in A:X ratio with further extraction steps in almost all species, consistent with previous literature (Cowley et al., 2021), although more sophisticated chemical analyses are needed. Moreover, it was found that the sum of HWF and IAE yield strongly correlates with water absorbing capacity (WAC), however content does not perfectly explain all of the differences in WAC so more research is needed linking chemistry with this property.

Niu et al. (2019) have studied interactions and functionality of whey protein and psyllium (1:2) prepared at different temperatures (room temperature, 60 °C, and 90 °C). Treatment at 90 °C resulted in a more uniform network, but it increased hardness, gumminess, chewiness, and resilience when compared to mixtures treated at 60 °C. The authors explained this as higher protein denaturation at 90 °C – exposed amino groups of protein could lead to pronounced carbonyl ammonia reaction, which favours homogeneous structuring and, therefore, increased hardness. However, according to the overall rheology profiles of fractionated *P. ovata* mucilage discussed in Section 3, higher extraction temperatures result in higher storage moduli (which relates to hardness of a material). Thus, such texture profile could instead easily be a consequence of the different temperature treatment of the psyllium fibre. Texture of the psyllium fractions alone were not shown. Nevertheless, psyllium-protein interactions, especially using a wider range of fractions, should be the subject of future research.

Fractionated psyllium gel can be added into gluten-free pasta as a structural network component (Fradinho et al., 2020). When comparing the best processing conditions of psyllium, it was determined that psyllium husk (160-315 µm particle size) treated at 40 °C resulted in pasta with desirable properties. Assessed temperature treatments showed that a 20 °C gel did not reach full maturation, while the others (40, 60, 80, 90 °C) did, and they showed weak gel-like structures and lower firmness. However, pasta incorporating a 40 °C gel had lower adhesiveness (which is a negative trait in pasta). Overall, the pasta that incorporated psyllium gel and rice flour (50:50) had lower cooking loss, and reduced carbohydrate digestibility which lowers

glycaemic index. Possibly the gel treated at 40 °C could provide more structural integrity than the gel treated at 20 °C, but also had desirable water holding and gel properties, which are lower than gels treated at higher temperatures.

The interaction of fibrillated cellulose (FC) and psyllium (PSY) heteroxylan fractions has been explored by Ren et al. (2020b). It was shown that FC-PSY composites have potential for incorporation into food products to obtain targeted rheological properties. Fluorescent microscopy showed that a psyllium fraction extracted at 60 °C (PSY-60) was affixed to FC, while other fractions (PSY extracted at 20, 60, and 80 °C, and whole psyllium husk) did not show association to FC. The authors attributed this phenomenon to the highly branched structure of PSY-60 which could have stereochemical compatibility with FC allowing interactions by hydrogen bonding.

Fibre from fractionated *Plantago* mucilage has found applicability in emulsion fabrication. The self-healing ability of hot water (85°C) extracted *P. ovata* mucilage was found to be a key factor in oil-in-water emulsion stability (Zhou et al., 2022). When used above its gelling point (0.5%), *P. ovata* polysaccharides formed networks in which oil droplets were embedded, and which could survive high shear during emulsion preparation. Work published by Niknam et al. (2018, 2020) has shown applicability of ultrasound/hot water-extracted *P. major* polysaccharides (75 °C, 40 min) as an emulsion and foaming stabiliser. The addition of *P. major* polysaccharides (0.3–1%) increased emulsion storage stability, heat and freeze-thaw stability, and decreased creaming (separation of emulsion phases). Foaming capacity decreased, but foaming stability increased with higher polysaccharide content.

Studying fermentability of different *P. ovata* fractions as opposed to whole psyllium husk has given valuable insight which might elucidate possible answers to seemingly contrasting results. Marlett & Fischer (Marlett & Fischer, 2003) have described three isolated psyllium mucilage fractions: A – alkali insoluble, B – alkali insoluble and acid soluble, and C – alkali and acid soluble. Fraction A had the highest amounts of arabinose and was not fermentable. Fraction B (55 % of psyllium mucilage) formed gels, was poorly fermented, and was the fraction responsible for increases in stool moisture and bile acid extraction, which lowers blood cholesterol levels. Fraction C (< 15 % of psyllium mucilage) is viscous and rapidly fermented. The authors attributed

differences in fermentability to different structural properties and gel forming potential of fractions, as well as “yet unidentified structural features that hinder fermentation by typical colonic microflora”. Recently, Harris et al. (2023) have compared fermentability of three *P. ovata* mucilage fractions (F1 – cold water extractable fraction, F2 – hot water extractable fraction, F3 – alkali extractable fraction) in *in vitro* conditions. The fractions show specific rheological profiles; F1, a viscoelastic fluid, F2, a weak gel, and F3, a true gel. F1 had higher amounts of galacturonic acid and rhamnose, while F2 and F3 had virtually identical monosaccharide and linkage composition. However, it should be noted that the hydrated psyllium fractions (0.5 g in 81 mL serum) were homogenised with an ultra-turrax for 3 minutes to allow for sampling, which could have reduced the gelling capacity. This allows an understanding of how gelling effects fermentability, while keeping other variables (mainly linkage, which is known to influence fermentability) consistent, but potentially does not reflect *in vivo* process. Moreover, this could provide answers for some of the inconsistent findings in earlier fermentation studies about psyllium. The highest total gas was produced by F1 (34.27 mL), and the lowest by F3 (3.00 mL). This indicates that the low viscosity of F1 enabled the intestinal enzymes to reach the substrate. The main metabolites of all three fractions was acetate, followed by propionate, and butyrate (approximate ratio for fractions and husk was 57:36:7). Interestingly, F2 and F3 had a very similar metabolite profiles. The authors concluded that gas production is related to viscosity, while linkage composition and sidechain motif affect SCFA output.

Altogether, using fractions of psyllium will affect product quality in different ways. A simple cold and hot water extraction will yield fibres with different properties. For example, one could choose cold water extracted psyllium when trying to achieve a food structure which is more viscous and less gel-like (such as in beverages), or when lower WAC is beneficial for texture (such as in bread). The higher fermentability of cold water extracted psyllium compared to raw psyllium and other fractions may also be desirable in products. A hot water fraction could be used when fibre type is important (i.e., when one would want only HX, without a pectin component) or when trying to achieve “solid” gel structure, perhaps in jellies or set yoghurts. The

development and optimisation of tailor-made blends of fractions for targeted properties could be a rich area for future studies. Finally, fractionation offers opportunities to use “clean” fibre which are not modified or treated, as such additives may have negative consumer perceptions (Kajzer & Diowks, 2021).

5.3. Whole seed flour

P. ovata is mostly used for husk production, with 75% of seed yield not being utilised, thus making its production hugely wasteful (Cowley et al., 2021). *P. ovata* seed composition is shown to be highly nutritious and less viscous. For this reason, whole seed flour (WSF) could be used to improve nutritional and functional profile of food products.

Noguerol et al. (2022) have compared a wide range of physicochemical properties of *P. ovata* husk (*Plantago* husk, PH) and *P. ovata* WSF (*Plantago* powder, PP). When compared to PH, PP had higher protein (2.5 vs 6.55%), lipid (0.5 vs 1.8%), mineral (2.1 vs 3.1%), and soluble fibre content (0.9 vs 42.1%). Moreover, PP had higher total phenolic content and antioxidant activity (900 mg GAE/100 g and 126 mg TE/100 g, as compared to PH which had 55 mg GAE/100 g phenolic content and 52 mg TE/100 g antioxidant activity). As expected, PH had higher water holding capacity and water retention capacity, while PP was more soluble. This indicates that PP could be easier to implement into food products. However, PP had a darker colour at all tested concentrations (1-7%), and the heating treatment (at 65 °C) resulted in increase in darkness, redness, and yellowness (Noguerol, Igual, et al., 2022). Overall, it seems that *Plantago* powder (from whole seeds) provides nutritional benefits, however both samples had certain functional advantages and disadvantages.

WSF from *P. ovata* and *P. psyllium* was compared with psyllium husk substitution (4 and 8%) in wheat bread (Pejcz et al., 2018). Psyllium husk substitution at both levels increased crumb moisture and volume, while WSF from *P. psyllium* had a negative effect on bread volume. Incorporation of both WSFs as well as husk led to darker crust colour, but there was no significant difference from the control in overall sensory acceptability. Bread with WSF had higher protein content, while bread with husk had higher dietary fibre content (Pejcz et al., 2018). Psyllium husk is a common addition in gluten-free breads, as it can improve its nutritional and techno-functional

properties (Belorio & Gómez, 2022). WSF was incorporated into gluten free rice bread – 5% of flour was replaced by either whole or ground *P. ovata* (PO) or *P. psyllium* (PP) seeds (Ziemichód et al., 2019). Replacement with whole PO and both types of PP seeds increased specific volume, but ground PO resulted in bread with specific volume lower than in the control. Both seed types had a protective effect on storage properties (hardness), with ground seeds showing lower hardness, which is desirable.

When psyllium husk (PH), psyllium husk powder (PW), and *P. ovata* seeds powder (PP) were incorporated into plant-based sausages (3-6%), it was determined that fibre addition resulted in improved nutritional profile (decreased fat content and increased ash and carbohydrate content) (Noguerol, Larrea, et al., 2022). Texture profile analysis has shown increased hardness when all fibres were added, but the lowest increase was in PP sausages. Water release was also the lowest in PP sausages, which is an important parameter for their quality and acceptance. However, sensory evaluation showed highest overall acceptability of plant-based sausages with PW (5 out of 7 score, whereas the control had the score of 3). Moreover, colour changes were the least prominent in PW sausages, while PP sausages had negative colour change and were evaluated the lowest on visual and colour score when compared to PH and PW sausages (Noguerol, Larrea, et al., 2022).

Detailed nutritional profile of WSF of 12 *Plantago* species, including *P. ovata*, has shown the potential to enrich foods with protein and unsaturated fatty acids, such as omega-3 and omega-6 (Cowley et al., 2021). *P. paradoxa* and *P. triantha* had >30% protein content, which is higher than chickpeas. A heart-healthy ratio of omega-3 to omega-6 for cardiovascular health is recommended to be 4:1 (Simopoulos, 2002); *P. turrifera* has shown said ratio to be 4.85:1, which is higher than flaxseed. Additionally, WSF from *Plantago* seeds does not contain starch, but mannans and fermentable oligosaccharides which were hypothesised to give WSF fermentative benefits over husk (Cowley et al., 2021). Indeed, when compared with husk in an *in vitro* fermentation study, WSF was better fermented than husk, with higher gas production and butyrate content, and lower propionate content (Harris et al., 2023).

5.4. Modifications

A book chapter by Yu et al. (2009) was published on health benefits of psyllium and approaches to improve its functionality, including changing the pH, hydrothermal treatments, conventional enzymatic treatments, solid-state enzymatic modifications, and chemical changes, such as grafting, networking, and alkaline treatments. Therefore, this section is an overview of psyllium modification papers published after 2009. In said book chapter, the authors described different modifications types used for psyllium and highlighted the usefulness of solid-state enzymatic reactions (L. Yu et al., 2003). In general, modifications reduced WAC and swelling capacity of psyllium while maintaining or improving certain health benefits. This poses a question: is high viscosity or the unique structure of psyllium responsible for physiological effects or can these two aspects be observed independently? The papers included below show that modifications can have different overall results which can be explored for greater benefits and ease of application. However, as the observed effects were discovered using *in vitro* assays, studies *in vivo* are necessary. Considering that health effects in people are explained mostly by high viscosity (Section 2), it is worth examining how psyllium with reduced viscosity would perform.

Modifications aimed at altering the functionality of *Plantago* species in health and food applications are listed in Table 2, although there are more describing pharmaceutical and engineering uses.

5.4.1 Physical modifications

Psyllium husk was processed by gamma radiation and used to make *dhokla* (Indian snack food) (Mallikarjunan et al., 2021). The husk was firstly extracted as water extractable and alkali extractable fraction (AEG), and it was reported that solubilization of AEG by gamma irradiation at different levels (5, 15, 25 kGy) was the contributing factor to overall husk solubilization. It was shown that *dhokla* fortified with processed psyllium husk received better sensory analysis scores than *dhokla* with native psyllium, which was described as gummy and chewy. Moreover, irradiated psyllium lowered sugar release in *in vitro* digestion when compared to unfortified *dhokla*, showing potential for developing foods with reduced GI. Positive effect of gamma irradiation on psyllium powder was reported for *chapati*, Indian

unleavened bread (Mallikarjunan et al., 2024). When irradiated, 14% psyllium incorporation in *chapati* was possible without negatively impacting its physicochemical and sensory properties, while un-treated psyllium was incorporated only up to 4%. *Chapati* with modified psyllium (14%) had low glycaemic index, while non-fortified and fortified with native psyllium had medium glycaemic index. Irradiated psyllium had higher solubility, which was caused by breakage of glycosidic bonds and chain scission (degradation of polymer main chain, which is a common occurrence in polymer radiation).

Polysaccharides from one of *P. ovata*'s relatives, *P. asiatica*, have been modified by high pressure homogenization (Hu, Nie, & Xie, 2013), microwave irradiation (Hu, Nie, Li, et al., 2013), and ultrasound (Huang et al., 2018). These processing methods have shown changes in polysaccharide structure and functionality, increased fermentability (Hu, Nie, Li, et al., 2013; Hu, Nie, & Xie, 2013), and increased anti-inflammatory activity (Huang et al., 2018), and thus could be viable options for modifying *P. ovata* fibre.

5.4.2 Chemical modifications

Using mild acid hydrolysis, psyllium husk was modified by HCl at different concentrations, and it was determined that HCl up to 1.44 % can reduce hardness and adhesiveness of psyllium gel without affecting its functional properties, such as bile acid binding (Cheng et al., 2009). This shows that mild acid treatment might be a promising candidate to change certain functional properties of psyllium, maintaining critical ones, while keeping the process inexpensive.

Chemical modifications, such as sulfation, hydroxypropylation, and succinylation have been employed to improve functionality of psyllium. Sulfation of psyllium resulted in weaker gel structure and lower swelling capacity, but higher water uptake and bile acid binding properties (Liu, Zhang, et al., 2010). SEM images showed changed structures after sulfation, which appeared grainy, possibly with a reduced surface area, unlike untreated psyllium, which was smooth. This change, as well as the introduction of negatively charged sulfonic groups, could have contributed to changes in the examined properties. Hydroxypropylation of psyllium husk revealed interesting results - two derivatives with a lower degree of hydroxypropylation (6.33

and 10.47 %) had enhanced bile acid binding properties and swelling capacities, while derivatives with higher substitution (12.63 and 15.26 %) had the lowest values of said properties (Liu, Xie, et al., 2010). In all cases, gel strength was reduced. Considering that the structure of all derivatives was similar, the authors concluded that the degree of substitution may have impacted binding capabilities. Succinylation of psyllium husk resulted in weaker gels, higher water uptake, and lower swelling capacity when compared to psyllium gel (Niu et al., 2012). Moreover, bile acid binding was increased in succinylated psyllium. Interestingly, three succinyl psyllium derivatives (with different substitution degrees – 1.9, 3.3, and 3.9) were not significantly different from each other when observing water uptake or bile acid binding capacity. Finally, the same research group has compared all three chemical modification methods (Niu et al., 2013). The important conclusion of these comparisons was that introduction of negative charges, size, and the steric effect of substitutions have different effects on functional properties of psyllium. Charge influences bile acid binding (sulfated psyllium had the highest capacity) and water uptake capacity (sulfated and succinylated psyllium had highest water uptake values). Altogether, the size of substitution groups could be the key to optimising gel forming ability and swelling volume in chemically modified psyllium.

A worthwhile observation on chemical modifications is considering if changed psyllium would be acceptable to consumers, who appreciate “clean labelled” food products (A. Chen et al., 2022). It is plausible that chemically modified psyllium would be scrutinised in the food market as modified celluloses and starches are, thus questioning its applicability beyond research.

5.4.3. Enzymatic modifications

Psyllium husk, partially hydrolysed by enzymes, was added to yoghurt before feeding it to Wistar rats for 90 days (Yadav et al., 2016). Both native and partially hydrolysed psyllium husk showed better lipid profile of blood plasma than conventionally prepared yoghurt and control groups, which shows that partial hydrolysis did not negatively impact hypocholesterolemic effect of psyllium. However very low-density lipoprotein levels were higher for native psyllium. Furthermore, the prebiotic effect

was enhanced when partial hydrolysis was applied to psyllium as SCFA content and lactobacilli count was higher in rats fed with modified psyllium.

5.4.4. Combined modifications

Psyllium husk was ball milled and then extracted by four protocols to obtain AXOS with different degrees of polymerization (DP) and A:X ratios (Pollet et al., 2012). For the first step of modification, extensive ball milling was conducted for 48 h. After this, the ball milled fibre was extracted under different pH conditions and temperatures. *In vitro* fermentation showed that all AXOS preparations were fermentable, and it was noted that long ball milling increased the fermentability of psyllium husk. AXOS with the lowest DP and A:X ratio showed the highest degree of fermentation. Enzyme activity of arabinofuranosidases, xylanase, and xylosidase were increased, as well as acetic, propionic, and butyric acid concentrations. It was suggested that this combined treatment can result in physiologically active AXOS and was recommended for use in psyllium husk modification where high WAC can present a challenge in food and health.

Table 2. Modifications applied on *Plantago* polysaccharides and subsequent results. Table includes type of modification, brief protocols explanation, and main results from papers about *Plantago* fibre transformation published after 2009.

Type of modification	Polysaccharide source and modification protocol	Results
Physical – gamma radiation (Mallikarjunan et al., 2021)	Psyllium husk processed by gamma radiation at 5, 15, 25, and 50 kGy	• Solubility was increased proportionally to radiation dose • Molecular weight, swelling capacity and gel strength were decreased • Bile acid and glucose retardation index were lower for irradiated psyllium than untreated • Control <i>dhokla</i> (Indian snack food) had higher sensory acceptability than <i>dhokla</i> fortified with treated psyllium, but textural parameters were not significantly different • In vitro starch digestibility was lower for fortified <i>dhokla</i>

Physical – gamma radiation (Mallikarjunan et al., 2024)	Psyllium husk powder processed by dry state gamma radiation and 25 kGy dose was chosen	- Irradiated psyllium formed weaker gel than non-treated psyllium - Increase in water uptake capacity and dough weight of <i>chapati</i> was observed – increase was higher in irradiated psyllium due to higher concentration of fibre used (14% for irradiated vs. 4% for non-irradiated psyllium) <i>Chapati</i> with psyllium had lower hardness after baking and after 24h storage <i>Chapati</i> without psyllium and with non-modified psyllium had medium glycaemic index (68 and 63, respectively), <i>chapati</i> with irradiated psyllium had low GI (46)
Chemical – acidic (Cheng et al., 2009)	Psyllium husk was acid hydrolysed by HCl (0.36, 0.72, 1.44, 2.88, 4.5 %) for 5 days	- Acid treated psyllium had lower water uptake properties, and gel adhesiveness and hardness 0.36 % HCl treated psyllium did not lose its swelling capacity (measured with simulated intestinal fluid), but others did - psyllium treated with up to 1.44 % HCl was dose dependant, but 2.88 and 4.5 % HCl treatments were time dependent as well - Psyllium treated with up to 1.44 % HCl did not lose the ability to bind bile acids when compared to untreated psyllium, but higher concentrations led to loss
Chemical – sulfation (Liu, Zhang, et al., 2010)	Acid treated psyllium husk was sulfated by sulfur trioxide-pyridine agent in dimethyl formamide to obtain 3 different sulfated derivatives (SP1, SP2, SP3)	- Elemental analysis showed sulphur content (%): SP1 – 10.37, SP2 – 11.11, SP3 – 13.11 - Sulfated psyllium showed decrease in gel strength (G') and lower apparent viscosity (proportional to sulfation degree) - Sulfated psyllium had higher water uptake capacity, but swelling volume (measured with simulated intestinal fluid) of SP1 was lower, while SP2 had same swelling volume as the control - Sulfated psyllium had higher bile binding capacity than the control – SP1 had 6.5x, SP2 had 5.9x, and SP3 had 6.3x higher cholic acid binding capacity than the control
Chemical – hydroxypropylation	Acid treated psyllium husk was mixed	Hydroxypropyl content (%): HP1 – 6.33, HP2 – 10.47, HP3 – 12.63, HP4 – 15.26

(Liu, Xie, et al., 2010)	with isopropanol and sodium hydroxide, then, different amounts of propylene oxide were added to produce 4 different hydroxypropyl psyllium derivatives (HP1, HP2, HP3, HP4)	- Hydroxypropylation reduced gelling capabilities of psyllium – HP gels had lower hardness and adhesiveness - Swelling volume (measured with simulated intestinal fluid) of HP1 was the highest, while HP2 was not different than the control, and HP3 and HP4 had the lowest swelling volume - Water uptake capacity of all HP derivatives were not significantly different than the control - Binding capacity for cholic acid was the highest in HP1, while HP2 was not significantly different from control and untreated psyllium, and HP3 and HP4 had the lowest capacity - Binding capacity for chenodeoxycholic acid was statistically same for untreated psyllium, control, HP1, and HP2, while HP3 and HP4 had lower capacities
Chemical – succinylation (Niu et al., 2012)	Psyllium was succinylated with 3 different doses of succinic anhydride and tributylamine to create 3 succinyl psyllium derivatives with low, middle, and high level of substitution	- Succinyl substitution degree: low – 1.9, medium – 3.3, high – 3.9 - Succinylation reduced gel strength and adhesiveness in dose-dependent manner - Water uptake capacity was increased, but swelling volume (measured with simulated intestinal fluid) was decreased increased when compared to native psyllium - Water uptake capacity and swelling volume were reversely correlated to degree of succinylation - Succinylated psyllium had higher bile acid binding capacity than native psyllium, but they were not significantly different from each other
Chemical – sulfation, Hydroxypropylation, succinylation (Niu et al., 2013)	Methods described in 3 previous rows	- Degrees of substitution: sulfated psyllium (SFP) – 1.02, hydroxypropylated psyllium (HP) – 0.88, succinylated psyllium (SCP) – 0.79 - All 3 derivatives had lower gel hardness and adhesiveness than untreated psyllium - Amongst modified psyllium, SFP had highest values of gel hardness and adhesiveness, while there was no significant difference between HP and SCP

		- SFP and SCP had significantly higher water uptake capacity, while HP did not differ from control psyllium - Swelling volume (measured with simulated intestinal fluid) was reduced when chemical modifications were applied – HP had the lowest swelling volume, followed by SCP and SFP - Bile acid binding capacity: SFP > SCP > HP > psyllium
Biological – partial hydrolysis with Viscozyme L (Yadav et al., 2016)	Psyllium husk was partially hydrolysed by Viscozyme L (cellulase, hemicellulase, xylanase, arabinose, β -glucanase activities) in solid state reaction; yoghurt was supplemented with native and modified psyllium and Wistar rats were fed for 90 days	- Blood lipid profile (tryglicerides, total cholesterol, HDL, LDL, VLDL, atherogenic index) measured after 90 days showed significant differences in rats fed with native and partially hydrolysed psyllium when compared to rats fed with conventionally prepared yoghurt - Blood lipid profile (tryglicerides, total cholesterol, HDL, LDL, atherogenic index) measured after 90 days did not show significant differences between native and partially hydrolysed psyllium, except in VLDL level, which was higher in native psyllium group - Rats fed with partially hydrolysed psyllium had higher SCFA count than rats fed with native psyllium, and both of them had higher count than other groups - Higher lactobacilli and lower coliform count was found higher in rats fed with modified psyllium
Combined – ball milling and different extraction protocols (Pollet et al., 2012)	Psyllium husk was ball milled for 48 h (500 rpm) and further extracted using 4 protocols (different solvents, temperature, and pH) to get 4 fractions with specific DP	- DP from highest to lowest was: 300, 200, 88, and 72 - A:X ratio from highest to lowest was: 0.29, 0.27, 0.16, and 0.14 - Fraction with highest DP (300) was least fermentable (around 30 %) - Fermentation with lowest DP was the most fermentable (50%) - Fermentation of fractions with DP of 200, 88 and 72 resulted in highest acetic and propionic acid concentrations - Fermentation of fractions with DP of 300, 200 and 88 resulted in highest butyric acid concentration

	and A:X ratios	

5.5. Genetic strategies

The genetics of xylan synthesis are difficult to study but the accessibility of psyllium's heteroxylan and synthetic tissue has made it useful as a model system to discover the underlying genes. A plethora of studies have implicated proteins in the GLYCOSYLTRANSFERASE (GT) families 43 and 47 in elongation of the xylan backbone and GT61 proteins in the attachment of side chains (Ye & Zhong, 2022). Supporting these, evidence from Expressed Sequence Tags and qPCR of developing psyllium integuments (containing the developing mucilage secretory cells) support the roles of GT43, GT47 and GT61 in psyllium seed mucilage heteroxylan synthesis (Jensen et al., 2013, 2014; Phan et al., 2016). Furthermore, suppression of GT43 and GT47 expression reduces xylan abundance (Lovegrove et al., 2013) and viscosity in wheat while several GT61s are responsible for adding substitutions in rice (Zhong et al., 2021), likely working in tandem, suggesting that xylan patterning and properties could be manipulated in *Plantago* by targeting these gene families. The criticality of side chains in the functional properties of psyllium makes GT61s an appropriate target. Overexpression or suppression of modifying enzymes in the mucilage secretory cells during heteroxylan synthesis are another possible strategy demonstrated recently in *Arabidopsis* (McGee et al., 2021). The recent genome sequencing of two *Plantago* species (*P. ovata* and *P. major*) (Herliana et al., 2023; Lyu et al., 2023) and emerging techniques for the stable transformation of *Plantago* species (Levengood et al., 2023) and changing legislation on genetic engineering and modification in foods makes the future of a genetic strategy for altering psyllium's functionality a promising one.

6. Conclusion and future directions

In this review, a comprehensive summary of the health benefits, mechanisms of action, and food industry uses of psyllium husk is presented. Psyllium addition is widespread in food products, fuelled by the aforementioned positive health effects, but certain challenges are evident.

Firstly, the inconsistent naming of *Plantago ovata* in scientific and popular literature should be mentioned. Husk from *P. ovata* is certainly the most commonly used in research and in food product development and preparation. The *P. ovata* husk is well known as ‘psyllium husk’, but sometimes confusingly is simplified to ‘psyllium’. This oversimplification leads to confusion in the literature as it was derived from the common name ‘psyllium’ which is used for multiple *Plantago* species, not just *P. ovata*. A great example is the plethora of literature concerning ‘*Plantago psyllium* husk’ or similar variations: *Plantago psyllium* (syn. *P. afra*) is a separate species from *P. ovata* historically also used as a supplement, but that cannot be husked in the same way (unpublished data). These literature are almost always referring to husk from *P. ovata*. Therefore, researchers must do their due diligence to provide a clear and detailed description of the materials used, including scientific name of the plant, specifying the part used (husk or the whole seed), and steps of preparation (for example, grinding). Specifically, if mucilage is used then all conditions of extraction(s) should be clearly stated, and it should be known if any insoluble parts of the husk are kept or discarded. The ‘psyllium’ is an established expression used in commercial settings and is unlikely to be changed, but it is recommended that researchers endeavour to identify/verify the botanical source of their materials (using a botanist or verified by the supplier), and include this in detail in the materials and methods, to ensure future reproducibility and easier interpretation of the literature. *P. ovata* husk, psyllium (*P. ovata*) husk, or even psyllium husk from *P. ovata* are sufficiently detailed alternatives to psyllium or psyllium husk alone.

Through detailed structural characterisation and rheological profiling, psyllium husk’s high viscosity and gelling capabilities are associated with several positive properties, but their successful implementation is likely to be closely linked to its application challenges.

Therefore, possible scenarios for broadening the industrial use of psyllium are proposed:

- 1) The use of other *Plantago* species presents a high chance to obtain subtle but discretely different polysaccharides that might act as a toolbox for targeted functionalities without the need for modification. Limited but promising rheology studies have shown similar but distinct functionalities that show promise to this end.
- 2) The use of fractionation might allow targeted isolation of fractions with specific properties. These fractions are extremely useful in research, and there is increased interest in their incorporation as food and health addition, but prolonged time and energy intensive isolation processes might hinder the transfer of the promising research in this area to commercial scales. Streamlining and upscaling of fractionation processes at pilot plant scale for commercial product development ventures are strongly recommended.
- 3) Whole seed flour (WSF) provides nutritional benefits, such as increased protein content and unsaturated fatty acids, as well as easier application in food products due to lower viscosity. In-depth studies on WSF should be carried out, with a focus on fermentability because of the mannan- and oligosaccharide-rich endosperm.
- 4) A wide range of modifications, including physical, enzymatic, chemical and combined, offer opportunities to change the structure of psyllium and influence its functional properties, namely the water absorption capacity and gelling behaviour. By introducing different functional groups, it is possible to change properties of the fibre, or choose enzyme(s) which would result in fibres with different structure. Importantly, modification-associated decreases in gelling might allow greater additions in food products, but the evidence suggest that the dietary fibre potency would be affected where gelling is altered. This would affect the accuracy of dietary fibre claims associated with recommended psyllium doses. More research should be conducted to assess the dose-dependence of dietary fibre benefits of modified psyllium and whether combinations might offer one practical solution to 'meet in the middle'.
- 5) Genetic manipulation could be exploited to target backbone patterning of psyllium fibre; thus, the distribution and length of sidechains, which are the main driver of gelling capacity, could be influenced. Additionally, enzymes encoded by *P. ovata*'s own

genome might also offer tools for an enzyme biotechnology approach where existing xylanase enzymes are ineffective at digesting psyllium heteroxylan.

Combined, bridging of the gap between relating chemistry and functionality of fractionated *Plantago* mucilage, recent research on understudied relatives of *P. ovata*, and newfound knowledge provided by *Plantago* genomics, has presented possible solutions to challenges arising when applying highly viscous mucilage into food products and as a health supplement. The utility of this review lies not only in identifying opportunities for targeted *Plantago* mucilage extraction and use, but also in providing potential new approaches when researching other myxospermous species.

Therefore, the aim of the thesis is to present research and address the existing gaps on the two described solutions: the use of alternative *Plantago* species and the use of fibre fractions. The mucilage of *P. lanceolata*, *P. turrifera*, and *P. drumondii* were compared to *P. ovata*. Mucilage of the species was fractionated using a 3-step method and composition and rheology of each fraction were characterised (chapter 3). The 3 alternative species have a wide range of viscosifying properties and represent varying comparison points for *P. ovata* mucilage. Further on, the fractions were applied to a starch-based system and their potential to improve the structure and modify digestibility was assessed. Mechanisms describing interactions between *Plantago* fibre fractions and starches are explored and proposed in chapters 4, 5, and 6, while the final research chapter (chapter 7) is focused on fibre fractions application in gluten-free bread.

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CHAPTER 3

Extraction, composition, and rheology of fibre fractions from four
Plantago species with varying hydration and gelling profiles

Title:

Extraction, composition, and rheology of fibre fractions from four *Plantago* species with varying hydration and gelling profiles

Authors:

Lucija Strkalj^{1,2}, Gleb E. Yakubov¹, Rachel A. Burton², James M. Cowley²

¹Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom

²School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

*Corresponding author

Highlights:

- *Plantago* mucilage exhibits diverse hydration, fractionation, and gel properties.
- *Plantago turrifera* and *Plantago drummondii* had the highest fibre yield and water absorption.
- *P. turrifera* and *P. drummondii* produced gels with exceptional resilience under deformation.

Abstract:

Plantago ovata (PO), better known as psyllium, is a mucilage producing plant that is widely used as food hydrocolloid, stabiliser, and texturiser in food industry due to its high gelling capacity. Other *Plantago* species, many of them mucilage producing, are considerably less explored and less utilised. Therefore, the aim of this work was to study fractionated fibre properties of *Plantago lanceolata* (PL), *Plantago turrifera* (PT), and *Plantago drummondii* (PD) and compare them to PO. All species had heteroxylan fibre with minor pectin component, and varying arabinose:xylose ratio, as confirmed by monosaccharide analysis and FTIR. PL showed low mucilage

content, while PT and PD had higher mucilage content and water absorption capacity than PO. Dynamic oscillatory rheology measurement were performed on PO, PT, and PD fibre fractions. Frequency sweep showed a range of viscoelastic behaviour: outer mucilage fractions from all seeds were viscoelastic fluids, and most other mucilage fractions were weak gels, except for PD fraction, which was a strong gel. PT and PD fractions showed extremely high resilience under deformation, which could be attributed to arabinoxylan with high arabinose:xylose ratio, potentially indicating the degree of substitution. This study confirmed suitability of *Plantago* mucilage from PT and PD, in addition to PO, as valuable food hydrocolloid.

Keywords:

Plantago heteroxylans, gel fractionation, monosaccharide composition, FTIR, dynamic oscillatory rheology

Abbreviations:

A:X = arabinose to xylose ratio, C = cold water fraction, CP = crossover point, FP = flow point, FTI = flow transition index, H = hot water fraction, I = intense agitation fraction, PD = *Plantago drummondii*, PL = *Plantago lanceolata*, PO = *Plantago ovata*, PT = *Plantago turrifera*, YP = yield point

1. Introduction

Plantago ovata husk (or psyllium) has long been a subject of research due to its highly useful hydration properties (Noguerol et al., 2022) and numerous health benefits, such as glycaemic control in patients with diabetes mellitus type 2 (Feinglos et al., 2013), weight loss in overweight and obese individuals (Gibb et al., 2023), cholesterol reduction in hypercholesterolemic individuals (Jovanovski et al., 2018), and stool normalising effect in individuals with constipation and IBS (Gunn et al., 2022; Jalanka et al., 2019; Major et al., 2018; McRorie et al., 2021). Its high water absorption capacity (27 g water/g psyllium husk) (Noguerol et al., 2022) and utilisation has been a subject of much research (Belorio & Gómez, 2022; Franco et al., 2020; Yu et al., 2009; Zhang et al., 2019). In an attempt to uncover mechanisms that drive its high viscosity and hydration, studies on the structure of psyllium polysaccharides have been reported, including its monosaccharide and linkage composition, studies on the impact of extraction protocols, and detailed rheological characterizations (Fischer et al., 2004; Guo et al., 2008; Ren et al., 2020; Yu et al., 2017, 2018, 2019, 2021). Psyllium husk is a dried fibre layer at the surface of the seed, which, when in an aqueous environment, absorbs water, swells, and forms mucilage (Cowley & Burton, 2021; Phan et al., 2020; Phan & Burton, 2018). When comparing its husk development and mucilage expansion mechanism, *P. ovata* is quite different than other known myxospermous (mucilage-producing seed) species, such as *Arabidopsis thaliana*, flaxseed (*Linum usitatissimum*), and chia seed (*Salvia hispanica*) (Cowley & Burton, 2021; Phan & Burton, 2018). In mature seeds, mucilage secretory cells (MSC) of *A. thaliana* and flaxseed rupture when in contact with water, release hydrated and swelled mucilage, and the remains of ruptured MSC can be easily detected. Contrastingly, *P. ovata* husk (which will become mucilage when hydrated) is independent of the MSC structure at maturity, instead simply expanding away from the seed when wetted after which the MSC remains cannot be recognised (Cowley & Burton, 2021). Fundamental science of the mucilage polysaccharide synthesis and formation has mostly been researched on *A. thaliana*. However, *A. thaliana* has suboptimal commercial value. Due to their higher volume of mucilage production and health benefits, psyllium, chia, and flax are more commercially widespread (Dybka-

Stępień et al., 2021; Goksen et al., 2023). *P. ovata* is unique in that its seed coat can be easily removed from the seed as part of the threshing process via dry stone milling, which allows for more convenient transport and storage than transporting the whole seed. The differences between *P. ovata* and other mucilaginous seeds do not end there. Monosaccharide composition analyses have shown that *A. thaliana* is mostly comprised of pectin with minor (though functionally critical) components like cellulose and xylan (Phan & Burton, 2018a). Flaxseed mucilage is characterised by approximately equal amounts of xylan and pectin (Phan & Burton, 2018), and chia mucilage is incredibly complex containing several unique polymers (Ang et al., 2023). Contrarily, psyllium consists of highly branched heteroxylan and minor pectin component (Cowley et al., 2021; Fischer et al., 2004; Guo et al., 2008; Phan et al., 2020; Yu et al., 2017). This composition is more similar to cereal arabinoxylans, however psyllium heteroxylans do not have ferulic acid residues and functionally they are dissimilar (He et al., 2021). For example, the arabinose to xylose ratio (A:X), which can indicate branching degree, for psyllium heteroxylan is reported to be 0.22-0.36, depending on extraction process (Cowley et al., 2021). On the other hand, A:X of wheat and corn bran is 0.6, and A:X of oat and rye bran is 1.0 (He et al., 2021). Even though the viscosifying ability is important for all arabinoxylans, psyllium, is shown to have much higher viscosity than cereal arabinoxylans – for example, 2 g of psyllium/100 g water has 48.80 Pa*s viscosity, while same amount of oat bran has 1.36 Pa*s (Pavlovich-Abril et al., 2012).

Physico-chemical properties (such as molecular weight, degree of branching, pH, molecular shape, functional groups) affect the extractability of a fibre (Maes & Delcour, 2002). Therefore, extraction protocols yield fibre fractions with different physicochemical and functional properties. Since psyllium is highly viscous and it is difficult to separate mucilage from the seed or the insoluble husk residue, acidic, alkaline, or sequential extraction protocols have been utilised to extract the psyllium fibre. Guo et al. (2008) have extracted 3 fractions based on the water and alkali solubility. The fractions differed in uronic acids content, and it was proposed that the water extractable fraction had the most complex side chains, while alkali soluble fraction had the least complex side chains. Moreover, the authors hypothesised that

certain fractions had mixed linkage xylan backbone (1,3- and 1,4- β -glycosidic linkage). However, mixed linkage backbone has been a subject of debate and currently, scientific opinion states that the heteroxylan backbone of *P. ovata* is composed solely of 1,4- β -glycosidic linkage (Cowley & Burton, 2021). Yu et al. (2017) developed an extraction process yielding three distinct fractions also based on fibre solubility in water and alkali. The outermost layer of the mucilage was composed of heteroxylan with pectin, whose role is the primary expansion when the husk comes into contact with water, followed by two inner layers, that provide structural support when the mucilage expands (Phan et al., 2020). Rheology supports this – the outer layer is a viscoelastic fluid, while the two inner layers are viscoelastic gels (Yu et al., 2017, 2021). A similar extraction method was described in Cowley et al.'s work (Cowley et al., 2020), but using an intense agitation step to replace the tedious alkaline extraction, neutralisation and dialysis. This method produced chemically similar fractions to Yu et al.'s method and was further shown to be applicable to other mucilaginous seeds. Marlett & Fischer (2005) have patented their extraction protocol of psyllium polysaccharides, which started with dilute alkali solubilisation (0.2 M) and centrifugation (Fraction A – alkali insoluble), followed by an acidification step (Fraction C – acid soluble, viscous but not gel-forming fraction) and final centrifugation (Fraction B – gel fraction). Subsequent research uncovered differences in fermentability: Fraction A was not fermented, Fraction B was only partially fermented (~25%), and Fraction C was rapidly fermented (Marlett & Fischer, 2003). The authors suggested that the linear arabinoxylan chain is fermented until an atypical branching point which cannot be hydrolysed by human microbiota is encountered. Recently, research has uncovered further reasons for differences in fermentability. Harris et al. (2023) have utilised the three-step extraction based on Yu et al.'s work (2017) and studied the fermentability of fractions *in vitro*. Their results showed that gelling capabilities have a significant effect on fermentability. Fraction 1 (F1) was a non-gelling, viscous fraction and produced the most gas and short chained fatty acid (34.27 mL and 41.39 mM, respectively). Fraction 2 (F2) and Fraction 3 (F3) had highly similar monosaccharide and linkage composition, but F2 was gel-like fluid, while F3 could form self-supporting gel. F2 and F3 produced similar amounts of metabolites (28.91 and 26.72 mM, respectively), but gas production was significantly

lower in F3 (F2 – 11.17 mL, F3 – 3 mL) (Harris et al., 2023). Therefore, gel properties of fibre fractions have an important role in functional attributes.

As psyllium fibre fractions are explored and more practical qualities are developed and described, it becomes apparent that the other *Plantago* species are still vastly understudied and underrepresented. This is further highlighted by current psyllium supply chain issues where global production needs to expand rapidly to temper the demand. The *Plantago* genus comprises over 200 species, and more than 77 show myxospermy (Grubert, 1974), so it is, therefore, reasonable to assume that *Plantago* species other than *P. ovata* could have similar gelling properties and would be a satisfactory alternative in health application and food production. For example, polysaccharide from *Plantago ciliata* seed extracted at 60 °C has a similar A:X ratio (0.23) and is rheologically a weak gel, similar to *P. ovata* polysaccharides (Addoun et al., 2020). On the other hand, *Plantago notata* polysaccharide extracted at 60 °C, had a lower A:X ratio (0.097) and lower uronic acid content (4.9 %) than is reported for *P. ovata* (Alizadeh Behbahani et al., 2017). *Plantago asiatica* alkaline extracted polysaccharide was found to have an A:X ratio of 0.24 and somewhat higher uronic acid content (20.50%), but was characterised as a weak gel (Yin et al., 2016). A study published by Cowley et al. (2021) examined fractionated fibre and mucilage profiles of twelve Australian *Plantago* species and showed that fractionation and hydration capacity differences arise from differences in pectin and heteroxylan composition. While the highest fibre yield was found in *P. ovata* (23.84%), the highest water absorption was reported for species with lower yields like *P. turrifera* (22.20 mg water/mg seed vs. 13.08 mg water/mg seed). Overall, the research shows potential that other *Plantago* species could be used as hydrocolloids for food and health purposes.

Therefore, the focus of this work is the characterisation of the seed mucilage of four *Plantago* species: *P. ovata*, *P. lanceolata*, *P. turrifera*, and *P. drummondii*. Psyllium husk is utilised in food industry and its structure and rheology are well studied. It represents a comparison point for other, less studied *Plantago* species. *P. lanceolata* is widespread in Eurasia and naturalised in Australia and through the world, and grows in grasslands and other harsh environments (Abate et al., 2022). It is reported

that *P. lanceolata* has been used for traditional medicinal purposes, such as wound dressing, abdominal pains and as an antimicrobial agent (Abate et al., 2022). *P. turrifera* and *P. drummondii* are Australian native species, and are as such adjusted to arid and dry climates (Hassemer et al., 2019). Fig. 1 shows the distribution of the four *Plantago* species in the world. *P. ovata*, *P. lanceolata*, and *P. turrifera* have shown various hydration properties (water absorption capacities are 13.08, 3.73, and 22.20 mg/mg, (Cowley et al., 2021)) and likely represent a spectrum of different functional properties including gelling behaviour and rheology. There is some evidence that in the species including *Plantago*, mucilage properties including yield are directly influenced by life history and environment (Cowley et al., 2021; Teixeira et al., 2020). This relationship could then partially explain why WAC is so high in *P. turrifera* as it is native to very dry environments in Australia. However, there is no report of *P. turrifera* rheology profile. *P. drummondii* is the least researched species of those and, to the best of our knowledge, there is no study on its hydration capacity, gel structure, fractionation profile, or health benefits, though the anecdotal evidence of its drought tolerance and high seed yield warrant its investigation.

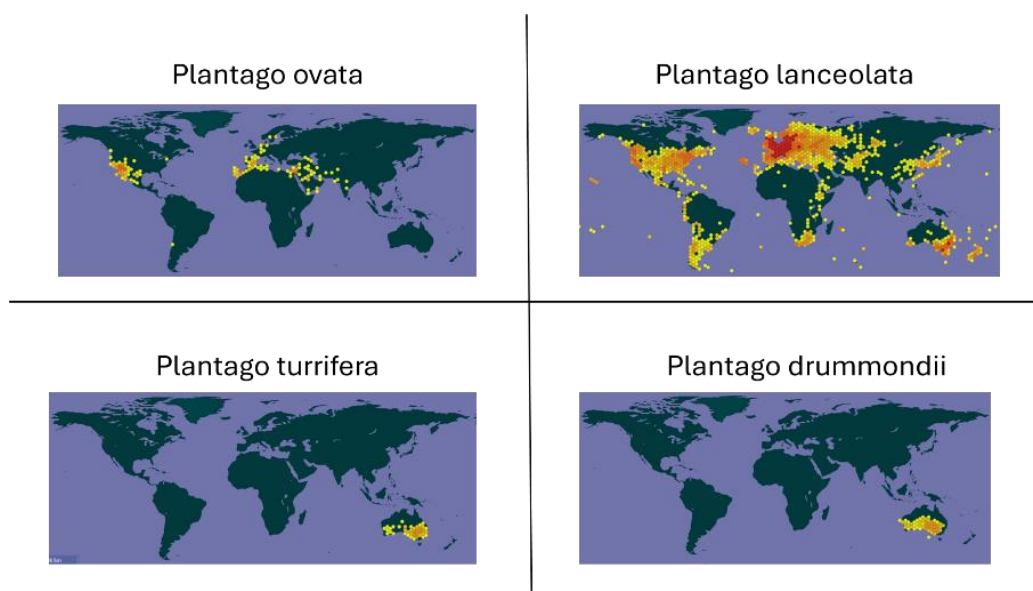


Figure 1. Distribution of *P. ovata*, *P. lanceolata*, *P. turrifera*, and *P. drummondii* in the world (GBIF, 2025).

As suggested in the previous chapter, two possible approaches to improve psyllium functionality are using underexplored *Plantago* species and extracting fibre fractions with different functional profiles. The 3-step fractionation used in this work yields fibre fractions which have different solubility and, consequently, different rheology profile. The first fraction is soluble in water at room temperature (cold water fraction – C), whereas the second fraction is soluble in water at a higher temperature (hot water fraction – H) and has viscous appearance at room temperature. The third fraction is not water soluble, but it is extracted via intense agitation as it is in gel form at the room temperature (intense agitation fraction – I). Utilizing these fractions derived from various *Plantago* species may facilitate the exploration of specific inquiries related to the application of psyllium, particularly concerning its targeted functionality, thereby closing the gap in the current understanding as potential hydrocolloid.

2. Materials and methods

2.1. Materials

Seeds from four different *Plantago* species were used to obtain fibre fractions: *P. ovata*, *P. lanceolata*, *P. turrifera*, and *P. drummondii*. Seeds of *P. ovata*, *P. lanceolata* and *P. turrifera* were originally sourced and produced as per Cowley et al. (2021), and where applicable, collection complied with relevant institutional, national, and international guidelines and legislation. Seeds of *P. drummondii* were originally collected on private land near Red Cliffs, Victoria, Australia.

2.2. Seed properties

2.2.1. Seed mass

Seed mass was determined by determining the mass of 100 seeds and dividing by 100. This process was done in triplicate.

2.2.2. Mucilage staining

To observe differences in mucilage architecture, whole seeds were hydrated in 0.01% v/w ruthenium red (C075, ProSciTech, Australia) prepared in RO H₂O. Solution was

vacuum sonicated for 10 minutes to remove bubbles. Each seed was placed on a microscope slide (Rowe GM2715, Australia), covered with a coverglass, and stained with a few drops of ruthenium red solution beneath the coverslip. The seed was left to hydrate for 10 minutes, after which the images were taken (Zeiss Stemi 2000-C, Germany, with a colour digital camera Zeiss AxioCam ERc 5s, Germany).

2.2.3. Mucilage fractionation

From each seed type, three mucilage fractions were extracted following Cowley et al.'s protocol (Cowley et al., 2020). Briefly, 30 mg of seeds were placed in 2 ml Eppendorf tube to which 1.5 ml of RO H₂O was added and shaken (1300 rpm) for 1.5h at 25 °C (Eppendorf ThermoMixer® Comfort, Germany). After brief centrifugation (10 000 rpm, 1 min, Eppendorf 5417R, Germany), upper layer was removed with 1 ml pipette without disturbing gel pellet and seeds. This layer is cold-water fraction (Fraction C). Small amount of RO H₂O was added to ensure that volume was 1.5 ml, as at the beginning of extraction. Similar process was repeated, however, the temperature at which the seeds were shaken was 65 °C, and hot-water fraction (Fraction H) was obtained. After re-adding RO H₂O, tubes were shaken (30 Hz, 20 min) using tissue-disruptor type mixer mill (Retsch MM400, Germany). Finally, after centrifugation, upper layer was removed from the seeds which is named the intense agitation fraction (Fraction I). Fractions were then stored in -80 °C freezer, and freeze dried to constant weight (Dynavac, Boyikang Laboratory Instruments Inc, China). Freeze dried fibre was used for subsequent experiments and to calculate fractionation profile and yield. To assess the yield of fibre based on seed mass, following equation was used (Equation 1):

$$\text{Equation 1: Yield (\%)} = \frac{\text{mass of freeze-dried mucilage}}{\text{mass of seeds before extraction}} * 100$$

Abbreviations used to refer to these fractions in the rest of the work will be specified by the source species and fraction, for example: PO-C is the cold water fraction from *P. ovata*. This considered, *P. ovata* = PO, *P. lanceolata* = PL, *P. turrifera* = PT, *P. drummondii* = PD, cold water = C, hot water = H, and intense agitation fraction = I.

2.2.4. Water absorption capacity (WAC)

WAC was determined according to Cowley et al. (2021), with minor changes. 30 mg of seeds were placed into 2 ml Eppendorf tube and 1 g of RO H₂O was added. After brief vortexing to break the surface tension, tubes were left undisturbed for 1 h. Unabsorbed water was removed with 1 ml pipette, whilst making sure that seeds and gel were not disrupted, and weighed. To calculate WAC based of seed mass, Equation 2 was used. To calculate WAC based on fibre mass, Equation 3 was used using the extracted fibre yield from Equation 1. Next, the WAC for dried fibre fractions was also determined. Protocol described above was followed, except that 15 mg of ground fibre (was used. Equation 4 was used to calculate the WAC for each fibre fraction.

$$\text{Equation 2: } WAC (g/g) = \frac{\text{Initial weight of added H}_2\text{O} - \text{weight of unabsorbed H}_2\text{O}}{\text{Weight of seeds before hydration}}$$

$$\text{Equation 3: } WAC (g/g) = \frac{\text{Initial weight of added H}_2\text{O} - \text{weight of unabsorbed H}_2\text{O}}{\text{Weight of extracted fibre}}$$

$$\text{Equation 4: } WAC (g/g) = \frac{\text{Initial weight of added H}_2\text{O} - \text{weight of unabsorbed H}_2\text{O}}{\text{Weight of ground fibre fraction}}$$

Additionally, images showing the swollen volume of hydrated seeds were taken following the same method as WAC. Instead of weighing the unabsorbed water, images were taken using a digital camera (MIUI Global 14.0.11, China) at a constant distance.

2.3. Mucilage composition

2.3.1. Protein content

Fibre solutions were prepared at 1 mg/ml in RO H₂O and used for protein content determination by Qubit Fluorometric Protein Assay Kit (Invitrogen, St. Louis, MO, USA) per manufacturer's instructions. Protein content was determined in triplicate.

2.3.2. Monosaccharide composition

Fibre solution (1 mg/ml) was prepared in RO H₂O. After acid hydrolysis (5M H₂SO₄, 100 °C, 2 h), fibre hydrolysate was derivatised with 1-phenyl-3-methyl-5-pyrazoline (PMP). Monosaccharide separation was performed by reversed phase high performance liquid chromatography (RP-HPLC) (Cowley et al., 2021). Area under the peaks was compared to standard curves of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose and fucose.

2.3.3. Fourier-transform infrared (FTIR) spectroscopy

Freeze-dried fibre was placed on diamond attenuated total reflection (ATR) crystal of an FTIR spectrometer (Alpha, Bruker, Germany). Spectra were collected in the range from 4000 to 400 cm^{-1} . The spectra were acquired averaging 128 scans with a resolution of 4 cm^{-1} against an empty background. Normalisation and baseline correction on whole spectra were performed by Opus 7.2.139.1294. Derivation was performed after smoothing (Savitsky-Golay, 1st order, with 20 neighbours) using Spectragryph 1.2. programme.

2.4. Molecular weight determination

Molecular weight determination was performed by size exclusion chromatograph (SEC, Agilent 1260 Infinity model, 1200 GPC/SEC MDS, equipped with Agilent PL aquagel-OH MIXED-H with 8 μM and the Agilent AdvanceBio SEC 300Å with 2.7 μM columns and AdvanceBio SEC 300A 2.7 μm 4.6x50mm guard column, Agilent, United States). Detectors were 1260 Infinity RI detector and 1260 Infinity Dual angle light scattering detector (Agilent, United States). Freeze-dried fibre (0.5 mg/mL) was dissolved in milli-Q H_2O with 0.5 mg/mL NaN_3 , and was placed in waterbath at 90 °C overnight to help with dissolution. The gel was then ultrasonicated (Qsonica Q500 sonicator, time – 30 s, amplitude – 12 μm , frequency – 20 kHz) to break down the aggregates. The gel was filtered through 0.45 μm filters before 50 μL was injected on the SEC. Mobile phase of SEC was milli-Q H_2O with 0.5 mg/mL NaN_3 , column temperature was 30 °C, and flow rate was 0.35 mL/min. Pullulan standards used had Mw range 180 – 1 540 000 Da (ReadyCal Pullulan High, PSS-PULKITR1H, Agilent, United States). Software used for parameters calculation was Agilent GPC/SEC Software version 2.2.281.39672 .

2.5. Dynamic oscillatory rheology

For dynamic rheology experiments, 1% w/v fibre was dissolved in RO H_2O , homogenised to remove potential aggregates (ProScientific, USA), and heated at 90 °C for 30 min while stirring with magnetic stirrer. After letting it cool to room temperature, fibre gel was loaded onto the bottom geometry (smooth lower parallel plate, 35 mm diameter, TMP35/Ti, Haake Mars, Germany) of air-bearing rheometer (Haake Mars iQ Air, Thermo Fisher, Germany). The upper smooth parallel plate (Haake

Mars P35/Ti, Thermo Fisher, Germany) was lowered to a gap of 1.2 mm to allow for trimming of fibre gel edges with a plastic spatula and covering the edges with mineral oil (330779, Sigma-Aldrich, Australia) to prevent evaporation during measurement. Final measuring gap was 1 mm. Two dynamic oscillatory measurements were carried out: frequency sweep and amplitude sweep. Linear viscoelastic range (LVER) determination was firstly determined to ensure the measurements within the LVER. Temperature was kept constant at 25 °C by a heat exchanger (Haake HX-R, Thermo Fisher, Germany). Frequency sweep was measured from 62.83 rad/s to 0.6283 rad/s under constant deformation (shear strain 0.03). Amplitude sweep was measured under constant frequency of 6.283 rad/s to measure the crossover point. All measurements were carried out in triplicates. All data were analysed using RheoWin 4.91.0011. software.

Storage (G') and loss (G'') moduli of frequency sweep were fitted to power law model using Equations 5 and 6:

Equation 5: $G' = A' * \omega^{b'}$

Equation 6: $G'' = A'' * \omega^{b''}$

G' and G'' are storage and loss moduli, ω is angular frequency, A' and A'' are intercepts at $\omega=1$ rad/s, b' and b'' are slopes. Goodness of fit (R^2) is also reported.

2.6. Statistical analysis

All data presented were determined in triplicate and presented as the mean and standard deviation, unless otherwise stated. All graphs were made using GraphPad Prism 9.0.0. (121) software, unless otherwise stated. Data were analysed in OriginPro 2022b. Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test ($p<0.05$). Correlation analysis was determined by Pearson's correlation ($p<0.05$). Power law analysis for rheology frequency sweep data (G' and G'') were determined in GraphPad prism 9.0.0. (121).

3. Results and discussion

3.1. Plantago seeds and mucilage properties

In this work, four different *Plantago* species were studied: *P. ovata* (PO), *P. lanceolata* (PL), *P. turrifera* (PT), and *P. drummondii* (PD). Due to their hydration and mucilage producing capabilities, they presented a spectrum of mucilage-related properties, and, therefore, were the species of interest in this work. Fig. 2A shows hydrated and swelled seeds stained with ruthenium red, which is a stain specific for acidic polysaccharides and is used commonly as a diagnostic stain for mucilage (Hou et al., 1999; Phan et al., 2020). PO, PT, and PD show a substantial mucilage layer surrounding the seed, while PL had less robust envelope relative to seed size. PO and PT architecture were consistent with the results presented here, with PD, like other Australian species, resembling PT closely with a wide unstained gel layer and thin outer layer compared to PO (Cowley et al., 2021). Kreitschitz et al. (2016) also studied *P. lanceolata* seeds, with their ruthenium red staining appearing similar to ours. Fig. 2A shows mucilage staining accompanied by mucilage:seed area ratio, which confirms that PL had the least amount of mucilage (1.63), and PT the highest (4.96). The mucilage:seed area ratio of PD was 4.04, and for PO it was 3.57. It is important to reiterate that PO is known for its high mucilage producing capacity, but the untapped potential of PT and PD, which produce even more mucilage, is shown here. PT seeds were the smallest and had the lowest mass (0.47 mg/seed), while PL had the highest (2.08 mg/seed) (Fig. 2B). PO and PT had very similar amounts of extracted fibre (20.3 and 20.8 % seed mass, respectively), while PD had 26.6% extracted fibre. In Cowley et al.'s (Cowley et al., 2021) work, PO had somewhat higher fibre content (23.84%), while PT had slightly lower fibre content (18%) which might be attributed to differences in seed stocks or in experimental handling. PO and its husk are known for high water uptake, which can be measured as water absorption capacity. Interestingly, when measured relative to fibre yield, both PT and PD seeds have significantly higher water holding capacity: PO – 69.88, PT – 114.08, and PD – 109.89 g/g. These results represent new fibre with extraordinary water uptake capabilities which are underexplored and underused. Water absorbing capacity based on seed mass agrees with previously published results, where water absorbing capacity of PO was 13.08,

PL 3.73, and PT 22.20 mg H₂O/mg seed (Cowley et al., 2021). Psyllium husk (not fractionated) has shown water holding capacity of 27 g H₂O/g husk and a water retention capacity of 25.8 g/g (Noguerol et al., 2022). Fractionation behaviour, related to differences in physiochemical properties that reduce extractability (Fig. 2C), reveals that mucilage from all *Plantago* species, except PL, had intense agitation fraction (I) as the most abundant. Particularly in PD, Fraction I represented 76% of the mucilage. This points to the importance of the innermost layer (I) which is anchored to the seed as necessary for WAC and gelling properties, and the integrity of the mucilage envelope. Proportions of fractions in PO, PT, and PL agrees with Cowley et al. (2021), and is similar to PO results in Yu et al. (2017). Next, the water absorption capacity of fibre fractions was determined (Fig. 2D). In general, Australia native species showed higher water absorption capacity than PO and PL, which agrees with the capacity determined for the seeds. PD-C had the highest water absorption capacity, followed by PO-H (41.38 and 39.08 g H₂O/g fibre, respectively), whereas the lowest value was measured for PL-I (5.51 g H₂O/g fibre). Interestingly, in almost all species, fraction I had the lowest water absorption capacity (except in PT, where all fractions had similar capacities). The reason for this occurrence could be the tendency of fraction I towards self-aggregation (Chen et al., 2024; Yu et al., 2017). Fractions H formed gels, but fractions C were soluble in H₂O at the room temperature, and most likely their water holding capacity is overestimated. The differences in their gelling profile (Ren et al., 2020; Yu et al., 2017, 2019) could explain the lack of clear trend when observing water holding capacity.

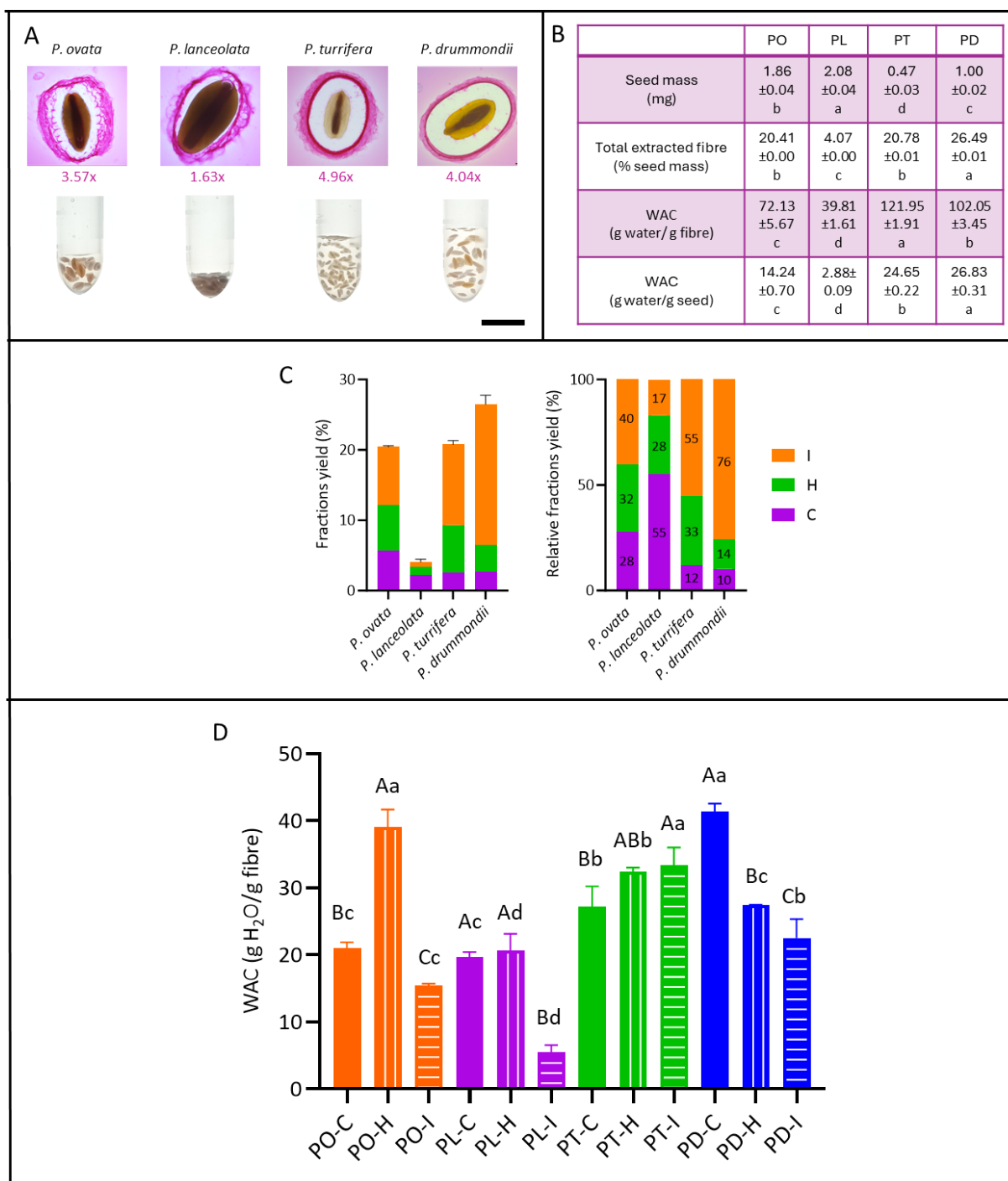


Figure 2. (A) Micrographs of hydrated *Plantago* seeds stained with ruthenium red. Below each image in pink text is the 2-D mucilage:seed area ratio. 30mg of seeds hydrated for 1.5h, after which excess water was removed. (B) *Plantago* seeds properties. Abbreviations: PO = *P. ovata*, PL = *P. lanceolata*, PT = *P. turrifera*, PD = *P. drummondii*, WAC = water absorption capacity. Different letters denote statistically significant difference ($p < 0.05$) between samples as determined by one-way ANOVA

followed by post-hoc Tukey test. (C) Yield (%) of fibre fractions based on seed mass. Relative fraction yield (%) represents proportion of each fraction based on mucilage. (D) Water holding capacity (WAC) of each fibre fraction. Different letters denote statistically significant difference ($p < 0.05$) between samples as determined by one-way ANOVA followed by post-hoc Tukey test. Capital letters represent significant difference between fractions of the same species, and small letters represent significant difference between same fraction of different species.

3.2. Monosaccharide composition and protein content

Monosaccharide analysis of the 12 extracted *Plantago* fibre fractions (Fig. 3) showed similarities in line with the previous work using these methods (Cowley et al., 2021; Yu et al., 2017). Namely, *Plantago* fibre are heteroxylans with a minor pectic component. Characterization of the pectin in *P. ovata* has been previously reported, and is described as unbranched rhamnogalacturonan (type 1) whose backbone consists of alternating rhamnose and galacturonic acid, and homogalacturonan, the type of pectin consisting only of galacturonic acid (Phan et al., 2016, 2020). Therefore, in this work, pectin is expressed as the summation of rhamnose and galacturonic acid determined by monosaccharide analysis, while the heteroxylan is expressed as the summation of arabinose and xylose. Relative monosaccharide composition is shown in Fig. 3A. From the monosaccharide data, the pectin to heteroxylan ratio (Fig. 3B), and the arabinose to xylose ratio (A:X) (Fig. 3C) are estimated, two factors that are likely to be critical to the fibre fraction functionality. Said ratios are important to illustrate changes following extraction steps and complexity of heteroxylans.

In all *Plantago* fibre fractions, the most abundant monosaccharide is xylose (from 60.263% in PL-H to 80.154% in PD-H). Arabinose is the second most abundant monosaccharide in most of other fibres (except PL-C, PL-H, PT-C and PD-C). *P. ovata* had a minor galactose component (3.242-4.461%). *P. ovata* showed decreasing amounts of pectin with further extractions steps. This agrees with published literature (Cowley et al., 2021; Phan & Burton, 2018; Yu et al., 2017). More precisely, pectin:heteroxylan ratios were 0.10, 0.02 and 0.00 in C, H and I, respectively. On the

contrary, the A:X ratio increased: PO-C and PO-H had similar A:X ratios (0.20 and 0.23, respectively), and PO-I had A:X ratio of 0.34. This trend is reported in Yu et al. (2017) and in Ren et al. (2020), and with glycosidic linkage analysis (Yu et al., 2017) points to the increasing sidechain substitution. *P. lanceolata* showed the most diverse monosaccharide composition, where galactose (10.528-13.109%), glucose (12.456% in fraction H), and rhamnose (6.770% in fraction C) had higher proportions than in the other *Plantago* species fractions. A reason for the high glucose content could be the cellulose fibrils, as they are thought to provide structural support for the mucilage envelope (Kreitschitz et al., 2016), though cellulose is generally resistant to the hydrolysis used in this work and linkage analysis found low levels of cellulose (Phan et al., 2016). Low molecular weight sugars are more probable explanation as these are abundant in *P. lanceolata* seeds particularly (Cowley et al., 2021) and the fractions were not dialysed. A known polysaccharide rich in glucose is β -glucan, but it is not typically reported for *Plantago*. Types of polysaccharides in *Plantago* plants have been discussed in the literature – a review on their bioactive properties has surmised that the seed polysaccharides are mostly arabinoxylans, while the leaf polysaccharides are pectin-like (Zhang et al., 2019). Hesarinejad et al. (2018) have reported 7.45% uronic acids in *P. lanceolata* mucilage extract (presumably extracted at room temperature; not specified), which is similar to the patterns identified here (6.665% uronic acids in PL-C, which was extracted at 25 °C). However, their overall monosaccharide composition does not agree with ours. The polysaccharide described in their work was described as an arabinoxylomannan, with high mannose content (69.42%), and lower arabinose and xylose contents (11.98% and 7.42%, respectively), which could be a consequence of misinterpretation of HPAEC-PAD peaks (as noted in chapter 2, section 5.1.) (Hesarinejad et al., 2018). The *P. lanceolata* monosaccharide profile agrees with other published literature, which describes heteroxylan with minor cellulosic and pectin component (Cowley et al., 2021; Phan et al., 2016). Potential explanation for high mannose content could be leaching from the seed endosperm, and the seed is rich in mannans (Cowley et al., 2021), but this is unlikely due to short extraction time (1 h). Moreover, their extraction protocol stated the first step as removing husk from *P. lanceolata* seeds by mechanical milling (Hesarinejad et al., 2018). This seems unlikely, as removable husk is a unique attribute of *P. ovata* (a

significant reason for its commercial use) (Cowley & Burton, 2021). Moreover, monosaccharide composition results of 12 different *Plantago* species have confirmed that they are heteroxylans (Cowley et al., 2021), thus *P. lanceolata* mucilage is described as a heteroxylan with minor components of pectin and cellulose. PL-C and PL-H had the same A:X ratio (0.13), and PL-I was somewhat higher (0.19). *P. turrifera* and *P. drummondii* had distinct monosaccharide profiles with mainly xylose and arabinose, but they did not have any galactose. PT-C and PD-C had a higher pectin:arabinoxylan ratio than in *P. ovata* (0.21 for PT-C, and 0.48 for PD-C, which was the highest in all species fractions). *P. turrifera* had a very apparent increase in the A:X ratio following extraction steps (0.05, 0.24, and 0.43 for PT-C, PT-H, and PT-I, respectively). This agrees very well with Cowley et al. (2021) where A:X increase shows the same behaviour. *P. drummondii* showed a slightly different trend in A:X ratio, where fractions C and H were equal (0.11 for both), and the fraction I had a much higher A:X ratio (0.59). In fact, PD-I had the highest A:X ratio of all the fractions tested here. PD mucilage fractionation is most similar to *Plantago varia*, per Cowley et al.'s work (2021), as they share very high A:X ratio of fraction I and they are both Australian native species. However, PD has significantly higher water absorption capacity (PD = 26.83, PV = 6.58 mg seeds/mg H₂O) and proportion of I in mucilage is much higher (PD = 76, PV = 47%) (Fig. 2). These findings could point to fraction I as a key to high WAC. In particular, the high values of A:X ratio points to dense sidechain distribution, which contributes to WAC and to the gelling capacity.

The protein content (Fig. 3D) was low in all species, as expected. In general, mucilage is high in fibre and low in protein (Cowley & Burton, 2021), as protein is mostly contained inside the seed. Noguerol et al. (2022) have reported 2.5% protein in psyllium husk, whereas *P. ovata* seed has 11.42% protein (Cowley et al., 2021). Farahnaky et al. (2010) examined composition of husk and mucilage, and protein content was lower than in the trends illustrated here and other reported results (0.25% for husk and 0.11% for mucilage). These discrepancies could be a consequence of different analytical method used or seeds from variable origin and storage conditions, which could impact overall composition and nutritional value.

As mentioned in the introduction, there is a significant lack of literature about *P. turrifera* and *P. drummondii*, due to them being underutilised Australian species. To the best of our knowledge, the only reported monosaccharide composition for *P. turrifera* is in Cowley et al.'s work (2021), and this work presents the first monosaccharide analysis for *P. drummondii*. The decreasing pectin:AX ratio and increasing A:X ratio with further extraction steps is seen in all studied *Plantago* species, pointing to the highly substituted AX being the key gel-forming polysaccharide. Thus, similar behaviour trends can be seen in the studied species, but the monosaccharide composition and the values of the ratios are different. This occurrence means there is variability between the species and the fractions, which could lead to variability in their gelling capabilities and, consequently, application in food and pharmaceutical industry.

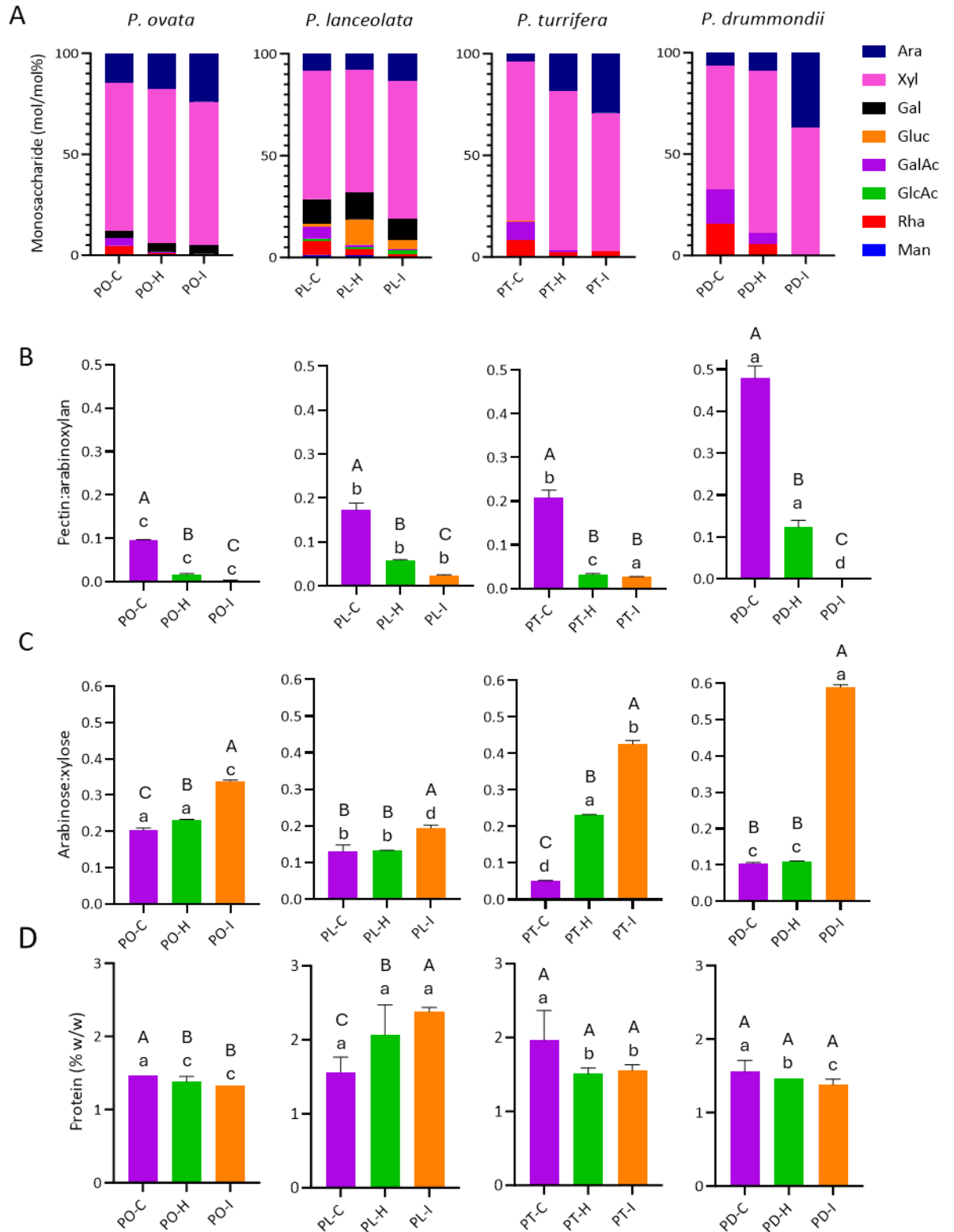


Figure 3. Monosaccharide composition (A) and corresponding parameters (B, C) of *Plantago* fibre fractions. Figure 3B shows pectin:arabinoxylan ratio of fibre fractions.

Pectin is calculated as rhamnose + galacturonic acid (%), and arabinoxylan is calculated as arabinose + xylose (%). Figure 3C shows arabinose to xylose ratio (A:X) of each fraction. Figure 3D shows protein content (% w/w) for each fraction. Different capital letters represent significantly different results between each fractions of the same *Plantago* species, while small letters represent significantly different results between the same fraction of different *Plantago* species. Both are determined by one-way ANOVA ($p < 0.05$) followed by post-hoc Tukey test.

3.3. FTIR

FTIR spectra of the *Plantago* fibre fractions are shown in Fig. 4. FTIR shows spectra typical for polysaccharides and is, in general, similar between the fractions and species. A pronounced broad peak can be seen from 3600 to 3000 cm^{-1} in all the fractions and represents O-H stretching vibrations, followed by a smaller peak at 2900 cm^{-1} which can be attributed to the -CH stretching vibrations (Patel et al., 2019). The peak at 1700 cm^{-1} is attributed to the C=O stretching in uronic acids (Ren et al., 2020). This peak is prominent in C fractions of all species, and in also PL-H. Moreover, it is particularly prominent in PD-C (Fig. 4D), agreeing with the monosaccharide composition results (Fig. 3). PD-C is the fraction with the highest galacturonic acid proportion (16.885%), and *Plantago* fractions showed the presence of uronic acids (mostly galacturonic acid) in C fractions. A similar trend was reported in CP and HP fraction from *P. ovata* (extracted at 25 and 80 °C, respectively) (Zhou et al., 2020). CP fraction had 18.50% uronic acids and defined peak at 1730 cm^{-1} , while HP fraction had only 3.50% uronic acids, and thus the corresponding peak was much smaller (Zhou et al., 2020). Another peak characteristic for ester carbonyl group of D-galacturonic acid is at 1370 cm^{-1} (Zhou et al., 2020), which is the most prominent for PD-C (Fig. 4D). The cluster of smaller peaks at 1500-1200 cm^{-1} is assigned to C-O-C glycosidic bonds vibrations (Ren et al., 2020). Besides assessing of the presence of monosaccharides associated with heteroxylan and pectin, FTIR can give us information about the arabinose to xylose ratio (A:X) in the fingerprint region of the spectra, which is from 1200 to 700 cm^{-1} . Wheat arabinoxylans (AXs) have been studied by FTIR and the second derivative spectra has indicated that two intensity of two

absorption peaks (984 and 958 cm^{-1}) and their ratio can provide us information about A:X ratio (Robert et al., 2005): the intensity of 958 cm^{-1} peak increased with the increase in arabinose substitution degree, while the intensity of the 984 cm^{-1} peak decreased (Robert et al., 2005). This finding provides a useful indication of the wheat AX structure, even though it is not the absolute value of A:X ratio (which can be determined by monosaccharide analysis). Therefore, this indicator is used as a complimentary analysis to *Plantago* heteroxylan monosaccharide composition despite there being certain differences between the *Plantago* fibre fractions and wheat AXs. While they both have the same backbone structure (β -(1 $\rightarrow$ 4)-linked xylopyranosyl units), the sidechains are quite different. Wheat AX sidechains are single α -L-arabinofuranosyl units, and xylopyranosyl units in the backbone can be unsubstituted, monosubstituted and disubstituted, although 60-65% of backbone units are unsubstituted (Robert et al., 2005). Contrastingly, the backbone of *P. ovata* is highly substituted, with only 18-20% units reported to be unsubstituted (Edwards et al., 2003). Sidechains in *P. ovata* are reported to be single xylopyranosyl side chain residues at O-2, single arabinofuranosyl units at O-2 and O-3, and the trisaccharide L-arabinofuranosyl-(1 $\rightarrow$ 3)-D-xylopyranosyl-(1 $\rightarrow$ 3)-L-arabinofuranosyl side chains at position O-3 (Edwards et al., 2003; Fischer et al., 2004). Moreover, the fractions that contain pectin and uronic acids have more complicated side chains (Edwards et al., 2003; Yu et al., 2017). Nevertheless, this approach is used to study A:X ratio in *Plantago* fibre fractions due to the previous reports of it being an effective complementary measure in *P. ovata* fractions. Ren et al. (2020) extracted five fractions from psyllium husk powder, at increasing extraction temperatures (20, 40, 60, 80 and 100 $^{\circ}\text{C}$). Reported A:X ratios calculated via second derivative peaks ratio (955 cm^{-1} /978 cm^{-1}) increased with increasing extraction steps (0.737, 0.884, 0.893, 1.63, 9.211, respectively), indicating increasing A:X ratio (Ren et al., 2020). However, in this study, second derivative spectra peaks and subsequent peak ratios did not produce the same trends as seen here. The reasons for that could be practical (lower peak intensity and definition, which would be caused by lower contact of the fibre sample and the FTIR instrument), or the fact that the data produced 2nd derivative values in the negative. Indeed, the absolute values of peak ratios behaved as expected (increased absolute value with increased extraction steps), however some of them

were negative (data not shown). Therefore, the analysis of the 1st derivative spectra was carried out, the peak definition and resolution was acceptable, and thus graphs and the calculated peak ratios (945 cm⁻¹/968 cm⁻¹) are shown in Fig. 4. The A:X ratio calculated by FTIR peaks ratio are significantly correlated with the A:X determined by monosaccharide analysis (Pearson r coefficient=0.82, p<0.05, Fig. 8). The highest peak ratio as determined by FTIR was 1.234 for PD-I, which also had the highest A:X ratio as determined by the monosaccharide analysis (0.590). However, it is important to note that the ratios seem to correspond when examining fibre fractions from a single species, but they do not correspond when examining all fractions from all four species. For example, PT-I has a higher A:X than PO-C when calculated from monosaccharide analysis data (0.462 and 0.203, respectively), but that is not the case in FTIR peaks ratios which were 0.675 for PT-I and 0.722 for PO-C. Therefore, this method seems to have limitations and should be interpreted cautiously. As explained, this could be due to need for determination of 1st derivative spectra due to low peak intensity, presence of other monosaccharide besides arabinose and xylose, or complexity of heteroxylan sidechains in *Plantago* mucilage.

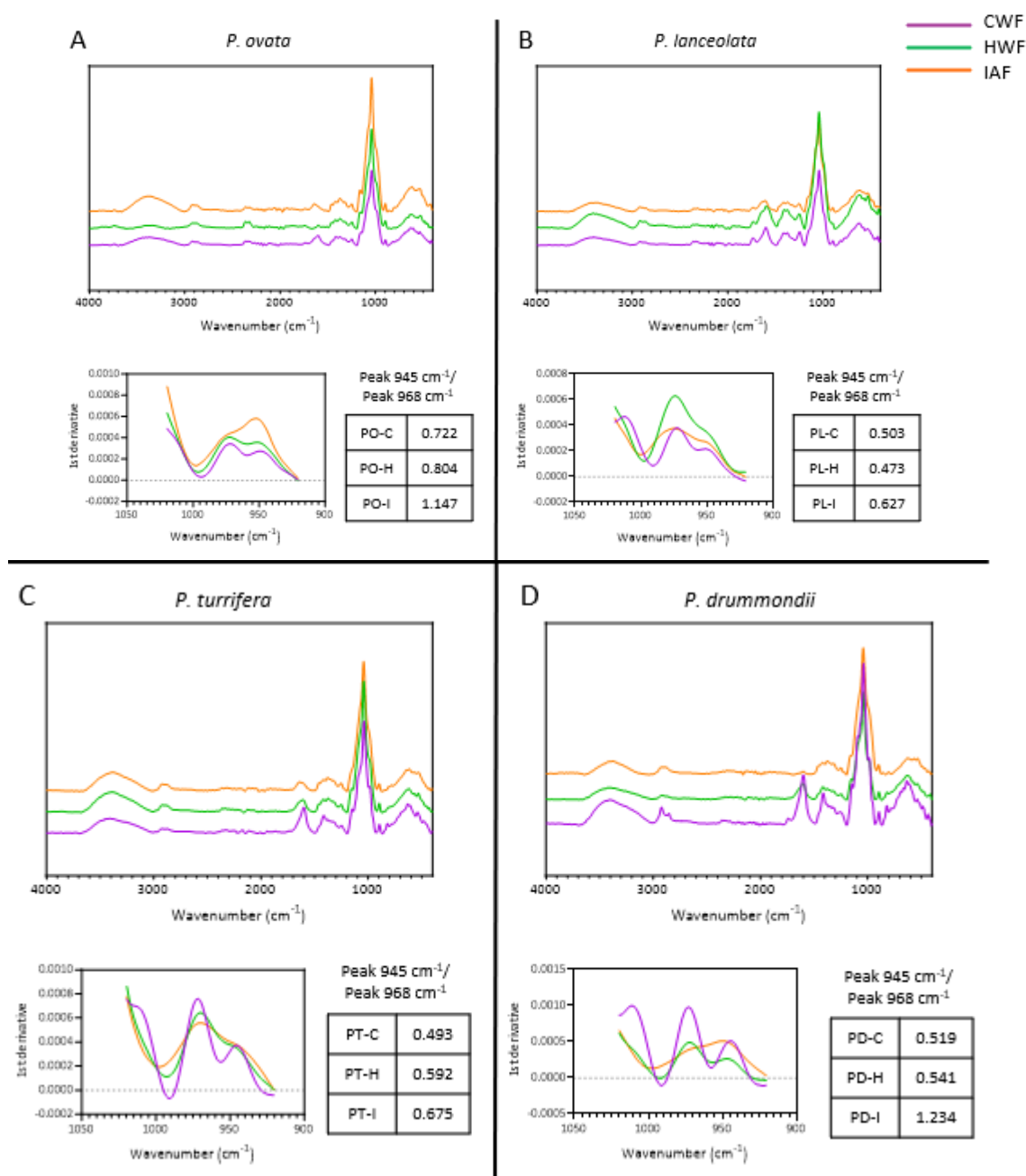


Figure 4. FTIR spectra (from 4000 to 400 cm⁻¹, larger panel) for all *Plantago* fibre fractions, 1st derivative of FTIR spectra (from 1020 to 920 cm⁻¹, smaller panel) and corresponding peaks ratio (845/868 cm⁻¹) representing A:X ratio. Purple colour represent CWF, green HWF, and orange IAF for each species. Figure 4A – *P. ovata*, 4B – *P. lanceolata*, 4C – *P. turrifera*, 4D – *P. drummondii*.

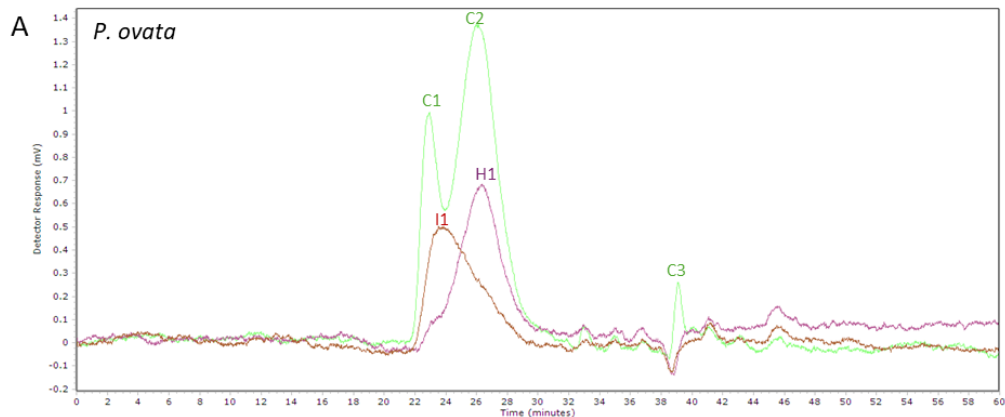
3.4. Molecular weight (Mw) determination

Molecular weight (Mw) determination by the size exclusion chromatography (SEC) and the properties of its corresponding peaks are presented in Fig. 5.

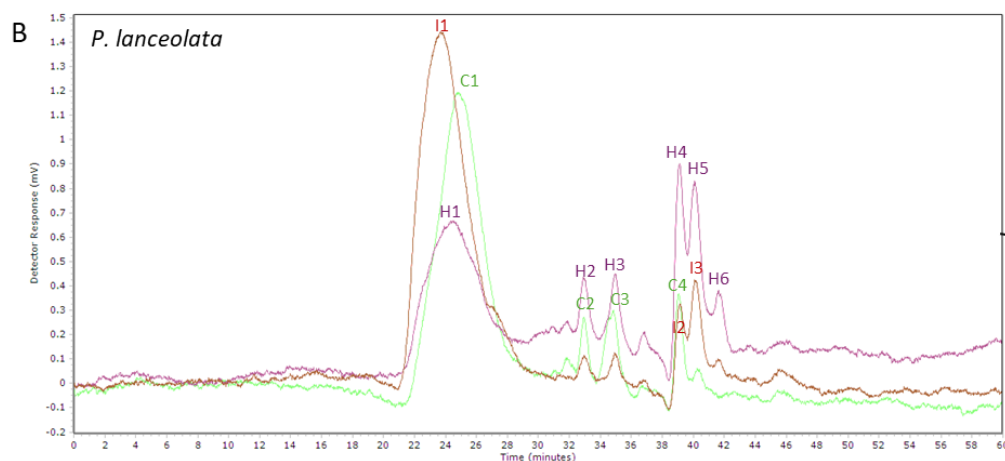
Studying Mw of *Plantago* fibres represents a significant technical challenge because of their high gelling capacity and viscosity. Usually, when studying Mw of fibres, the fibre concentrations of approximately 1-2 mg/ml are dissolved in the mobile phase and filtered through 0.2 or 0.45 μm filters, as shown in several examples from the literature like sugar beet pectin and citrus pectin (1.5 mg/ml in 100 mM NaNO_3 with 0.04% NaN_3) (Lin et al., 2024), *Mesona chinensis* Benth polysaccharide (1 mg/ml in ultra-pure H_2O) (Huang et al., 2018), or polysaccharides extracted from flaxseed hulls (1 mg/ml in 100 mM NaNO_3 with 5 mM NaN_3) (Qian et al., 2012). Previously, the Mw of *P. ovata* fibre fractions was determined by dissolving 2 mg/ml of fibre in 0.04 M KOH with 60 mmol/L CsCl, stirring overnight, ultrasonication, and filtering through 0.2 μm filter (Yu et al., 2017). Since PO fractions have been alkaline extracted, presumably alkaline solvent and mobile phase could solubilise fibre and lower its viscosity by acting as a chaotrope and preventing hydrogen bonding critical to gel formation (Yu et al., 2017, 2018). The SEC system used in this study (described in section 2.5) had certain limitations, namely that the pH of the mobile phase needed to be in pH=2-10 range. Therefore, alkaline solvents could not be used (for example, 0.04 M KOH has pH of c. 12.6). The mobile phase was ultra-pure H_2O + 1 mg/ml NaN_3 (to prevent microbial growth). The primary challenge was filtering the dispersed fibre through a 0.45 μm filter, since the fibre aggregates would completely block the pores, and the solution could not be filtered. Guanidine hydrochloride (GuHCl) was used, a chaotropic solvent proven to disrupt hydrogen bonds and decrease formation of a gel network (Yu et al., 2017). When dissolving the fibre fractions in 8 M GuHCl, the solution was indeed much less viscous, but still could not be filtered. Many solvents are known to disrupt hydrogen bonds - organic solvents like dimethyl sulfoxide and dimethylacetamide and salts like lithium bromide. However, the instrumental setup was limited (there were no fitting columns), so other approaches had to be explored. Nevertheless, using said solvents would be an advisable avenue to try. Next, the fibre concentration was reduced to 0.2 mg/ml, which could be hot-filtered (dissolved fibre

was left overnight in a hot water bath at 90 °C, and filtered without cooling), which was technically successful, but the peak response detection on SEC was too low. Therefore, the concentration was increased to 0.5 mg/ml, which again proved to form too large aggregates to be filtered. The next step was to try ultrasonication, which was more successful as the ultrasonicated fibre could be filtered, and peaks were detected (Fig. 5). This method, (described in the section 2.5), was used as the final method. Still, the results may not accurately reflect actual fibre Mw properties. Yu et al. (2017) have reported PO Mw values: CWF – 1085 kDa, HWF – 971 kDa, and KOH – 953 kDa (which corresponds to the Fraction I). They also found that hydrodynamic radius increases with increasing extraction steps (35, 42, and 45 nm for CWF, HWF, and IAF, respectively) (Yu et al., 2017). This is important, as the SEC is actually an instrumental technique that measures the size of molecules, whereas molecular weight is subsequently calculated (Irvine, 2000). On the other hand, Zhou et al. (2020) have found that CP fraction of *P. ovata* (extracted at 25 °C) has a lower Mw than the HP fraction (extracted at 80 °C) (6.3×10^5 and 2.1×10^6 g/mol), but the hydrodynamic radius increased (CP – 20 nm, HP – 33 nm). The results suggest that PO-C had the highest Mw, followed by PO-I, and PO-H (19274.4, 6046.02, 10809.2 kDa, respectively) (Fig. 5A). For PL and PT, the Mw increased with further extraction steps (PL: $9691.52 < 11637.2 < 13890.5$ kDa; PT: $12725 < 14836.1 < 17413$ kDa, for C, H, I, respectively) (Fig. 5B and 5C), and in PD, the C and H were similar (13462.3 and 12649 kDa, respectively) (Fig. 5D). However, these results should be interpreted with caution, as the technical limitations may have impacted the validity of the results. Due to the neutral pH of mobile phase, there is a possibility of fibre gel aggregates sticking to the column. Moreover, the dissolved fibre in this case most likely forms micro- and nano-gels, where aggregates are formed from multiple *Plantago* polysaccharide domains. The semi-aggregated state of entangled polymers is what is most likely being detected. The highest peak was detected in PO-C, and its corresponding Mw was 19 274.4 kDa (Fig. 5A). The largest reported protein molecule is called titin, which has Mw of 3000-3700 kDa (Rudolph et al., 2019). Therefore, it is logical to assume that gel aggregates are being detected.

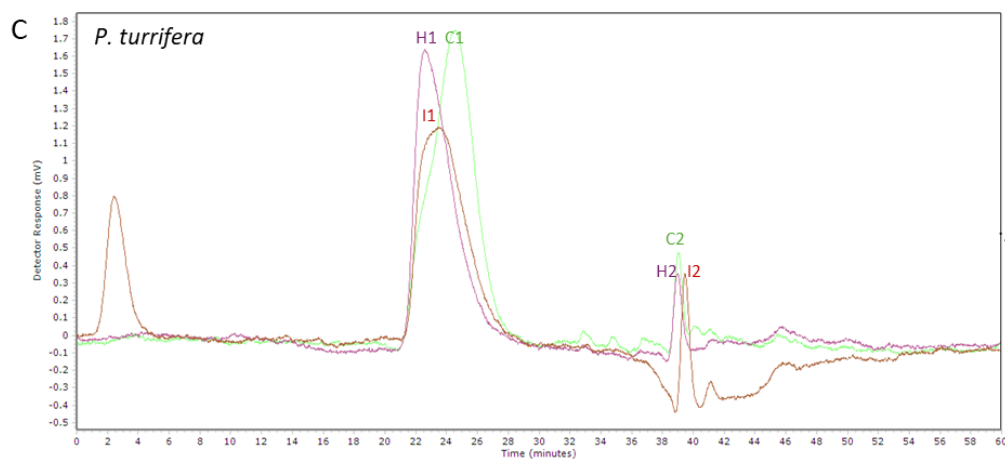
Overall, it is likely that the Mw reported here are not accurate due to the method and sample limitations. Nevertheless, the method development provided solid indications and direction for future experiments, where the next step will be to use SEC system compatible with ionic liquids which would enable thorough dissolution of *Plantago* polymers. This will be important for further characterization of *Plantago* fibre, as there are no reports on Mw of *P. lanceolata*, *P. turrifera*, and *P. drummondii*.



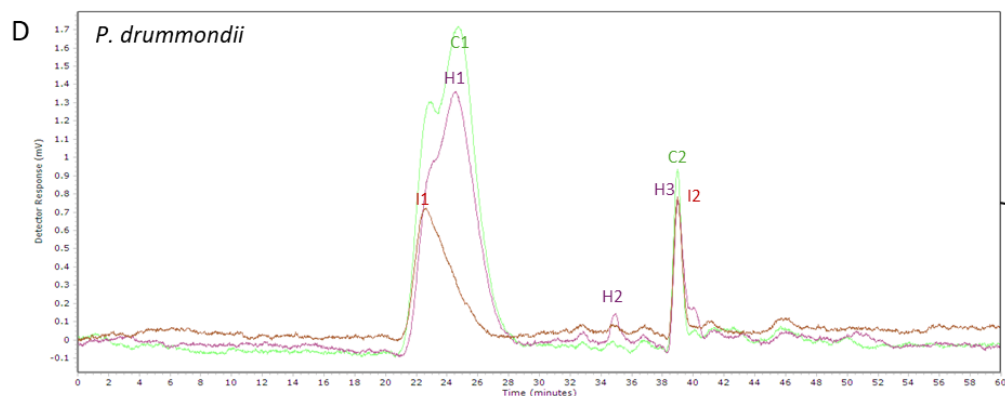
	Mw (kDa)	PDI
C1	19 274	1.246
C2	29 678	1.012
C3	5792	1.04
H1	6046	1.32
I1	10 809	1.49



	Mw (kDa)	PDI
C1	9 691	1.394
C2	372	1.026
C3	177	1.011
C4	30	1.027
H1	11 637	1.033
H2	383	1.009
H3	166	1.017
H4	30	1.033
H5	19	1.48
H6	11	1.012
I1	13 890	1.007
I2	19	1.02
I3	19	1.479



	Mw (kDa)	PDI
C1	12 725	1.009
C2	31	1.332
H1	14 836	1.003
H2	26	1.412
I1	17 413	1.006
I2	32	1.309



	Mw (kDa)	PDI
C1	13 462	1.011
C2	32	1.362
H1	12 649	1.034
H2	161	1.013
H3	29	1.316
I1	18 302	1.015
I2	31	1.213

Figure 5. Molecular weight determination by size exclusion chromatography and corresponding parameters (M_w – molecular weight in kilodaltons, PDI – polydispersity index). Fractions from all species are represented by same colour: C – green, H – purple, I – dark red. Figure 5A – *P. ovata*, 5B – *P. lanceolata*, 5C – *P. turrifera*, 5D – *P. drummondii*.

3.5. Rheology

Rheology, a study of flow, can provide insight into how soft materials, such as gels, behave under deformation. Rheology enables studying and predicting behaviour of a material in both scientific and commercial settings, including food science. Moreover, it facilitates better understanding of a material's structure and the interactions between its components. For gels, those components are a network (comprised of one type or multiple type of polymers) and a continuous phase (for example, H_2O) (Bashir et al., 2020). In this work, two rheology tests were carried out: the frequency sweep and the amplitude sweep. The frequency sweep is used to characterise the viscoelastic behaviour of a material by varying the oscillatory frequency. The parameters studied in frequency sweep are the storage (or elastic) modulus (G' , a measurement of material's ability to store energy elastically; representing the stiffness of a material), loss (or viscous) modulus (G'' , a measurement of dissipated energy; representing the flowability of a material), and $\tan \delta$ (G''/G' , which indicates whether material is behaving more like viscous or elastic matter and how the change in frequency affects the studied material) (Hyun et al., 2011). The values of $\tan \delta$ can also be used to indicate the type of gels: $\tan \delta > 1$ – viscoelastic fluid, $1 > \tan \delta > 0.1$ – weak gel (deforms under stress but still retains some structural characteristics), and $\tan \delta > 0.1$ – strong or true gel with the high degree of elasticity (Farahnaky et al., 2010). Strong gels usually have covalent or ionic bonds, whereas the weak gels can be described as physical gels whose network is stabilised by hydrogen bonds.

Dynamic oscillatory rheology measurements are presented in Fig. 6. Elastic and viscous moduli (G' and G'') and $\tan \delta$ determined by frequency sweep are presented in the Fig. 6A and 6B, while amplitude sweeps are shown in the Fig. 6C. G' and G'' of

frequency sweep fitted to a linearised power law equation are expressed in the parameters A' and A'' (intercept at $\omega=1$ rad/s), b' and b'' (slope), and R^2 (goodness of fit) are in Table 1. R^2 show that all data were well fitted to the power law model, with $R^2>0.9$. Amplitude sweep parameters can be found in table 2.

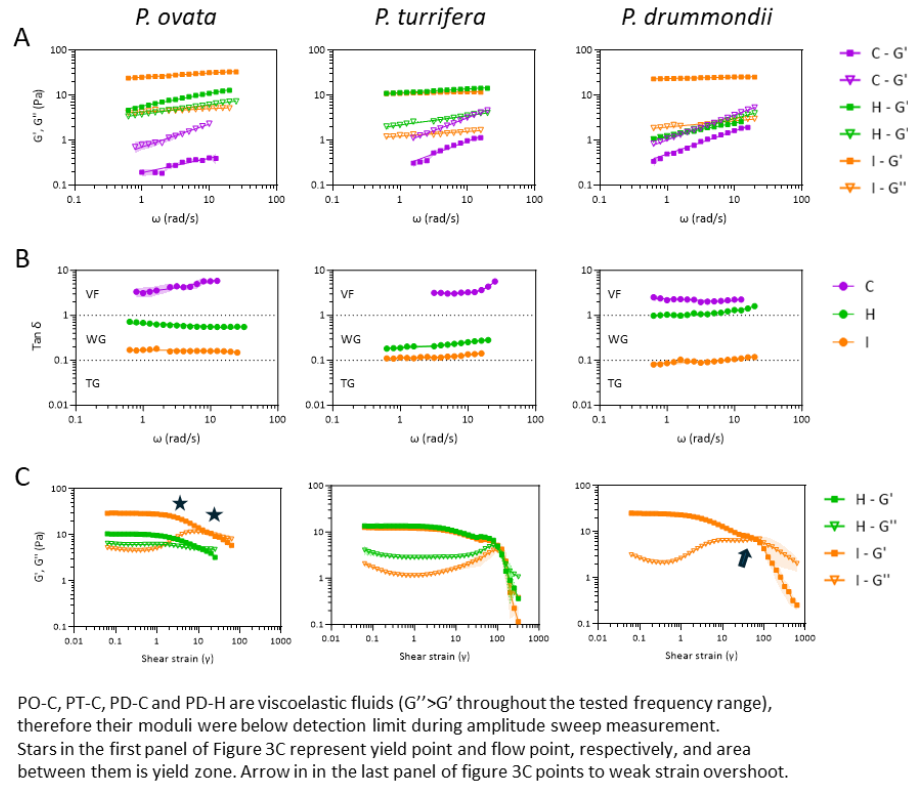


Figure 6. Rheology studies of 1% fibre fractions in water at 25 °C. Top row indicates species. Different colours represent fractions of all species: C – purple, H – green, I – orange. G' is marked by full squares, G'' is marked by empty downward triangles. $\tan \delta$ is marked by closed circles. Figure 6A: G' and G'' values determined by frequency sweep at constant strain (0.03). Figure 6B: $\tan \delta$ (G''/G') as determined by frequency sweep. Material type regions are divided by dotted lines where VF is viscoelastic fluid (>1), WG is weak gel (1-0.1), and TG is true gel (<0.1). Figure 6C: G' and G'' values of viscoelastic solids determined by amplitude sweep at constant frequency (6.283 rad/s).

The rheological studies on three species: *P. ovata*, *P. turrifera*, and *P. drummondii* are presented. *P. lanceolata* was not included due to the solution being liquid-like with visible clumps of insoluble fibre, and flow comparable to H₂O at 1% concentration (personal observation). A reason for low solubility could be the presence of the aggregates surrounding the cellulose fibrils (Kreitschitz et al., 2016), as cellulose is known to be insoluble in H₂O (Pinkert et al., 2010). A paper published on PL extract rheology described it as weak gel, with a G' of ~1000 Pa of 2% solution (strain sweep at 1 Hz frequency) (Hesarinejad et al., 2018). Certain doubts with this work are expressed in the section 3.2, as based on monosaccharide analysis, they reported PL polysaccharide as arabinoxylomannan, and the validity of the reported rheology results is discussed here. To compare, a 2.5% *P. ovata* gel showed $G' \sim 180$ Pa (strain sweep at 10 Hz) (Farahnaky et al., 2010). Another work comparing *P. lanceolata* and *Linum usitatissimum* (flax) mucilage has described *P. lanceolata* mucilage as having weaker adhesive and frictional properties (Kreitschitz et al., 2016), which indicates weaker properties when compared to *P. ovata* as well, as *L. usitatissimum* produces rheologically weaker mucilage than *P. ovata*. As already discussed, it is possible that Hesarinejad et al. (2018) misidentified the seeds at purchase and were either another *Plantago* species or another taxa entirely. Overall, the results presented here are chosen for three *Plantago* species which show high gelling potential.

The mechanical spectra, or frequency sweep results (Fig. 6A) and $\tan \delta$ throughout the frequency sweep (Fig. 5B), show that Fraction C of all species are viscoelastic fluids, with $G'' > G'$ ($\tan \delta > 1$) and frequency dependence. Parameters A' , A'' , b' and b'' (Table 1) were similar for all C fractions and also suggest frequency dependence. Frequency exponents are highest for PT-C ($b'=0.56$, $b''=0.57$), and PT-C showed the biggest difference between A' and A'' (0.26 and 0.84 Pa, respectively; A''/A' of 3.23), confirming the viscous nature of the material. Yu et al. (2017) and Zhou et al. (2020) have studied fraction of *P. ovata* extracted at 25 °C. Both reported viscoelastic fluid response for 1% dispersions, agreeing with the shown results. However, Zhou et al. (2020) also noted the appearance of a crossover point for G' and G'' at higher CP concentrations, which was a fraction extracted at 25 °C (3% CP had crossover point at ~30 rad/s, 4% CP had crossover point at ~9 rad/s), which points to the presence of

more and more entangled networks at higher concentrations. Fractions H display different rheological profile from each other. PO-H has $G' > G''$ in the tested range ($\tan \delta \sim 0.8$; A''/A' of 0.65) (Fig. 6A and 6B), however it shows slight frequency dependence ($b' = 0.28$, $b'' = 0.21$) (Table 1), making it a weak gel. Yu et al. (2017) have reported weak gel structure of *P. ovata* HWF, as their $\tan \delta$ was < 1 , and b' parameter was 0.4, which indicates frequency dependence. PO-H and PD-H show slightly different trends. PD-H, whose G' and G'' are almost equal throughout frequency sweep ($\tan \delta \sim 1$; $A''/A' = 0.96$) (Fig. 5A and 5B), has parameters $A' = 1.24$ and $A'' = 1.19$ Pa, and $b' = 0.29$ and $b'' = 0.41$ (Table 1), thus its viscous modulus shows higher frequency dependence than elastic modulus, and its behaviour can therefore be described as a viscoelastic fluid. Moreover, $\tan \delta$ values of PO-H and PD-H are similar, but their G' and G'' are different, which indicates that both of these fractions have comparable viscoelastic profile in terms of dissipated energy relative to the stored energy under oscillatory deformation, but their mechanical properties vary as the material with higher G' and G'' can recover its shape after deformation (Mezger, 2015). Practically, this would mean that these fractions could be used to achieve food products with similar texture, however under deformation they would behave differently and so their use would need to be adjusted during certain processes (such as mixing, kneading, blending, stirring, etc.), but may be advantageous in scenarios where PO-H's mechanical properties cause processing issues. On the other hand, PT-H is different from both PO-H and PD-H. PT-H is a viscoelastic solid ($G' > G''$, $\tan \delta \sim 0.2$; $A''/A' = 0.19$), and can be described as a moderately strong gel (Fig. 6A and 6B). Out of all H fractions, PT-H has the strongest gel properties. Parameter b' of PT-H is 0.08, which shows that it is frequency independent (Table 1). Its storage modulus (G') at 1 rad/s is higher than G' of PO-H and PD-H (parameter A' is 11.39, 5.76, and 1.24 Pa for PT-H, PO-H and PD-H, respectively), and $\tan \delta$ lower, indicating that under oscillatory deformation, most of energy is stored. Fractions I of all three species can be classified as viscoelastic solids that are frequency independent. Their G' are higher than G'' in the tested frequency range, and parameter b' is lower than 0.1 (PO-I – 0.09, PT-I – 0.03, PD-I – 0.03). The ratio b'/b'' can tell us about hydrogel behaviour – if the ratio value is 0, then a hydrogel is considered to be covalently crosslinked, and the higher values are reported for entangled hydrogels (Fan et al., 2022). The b'/b'' ratio of PO-I is 1.13, PT-

I is 0.27, and PD-I is 0.21, thus they are classified as entangled hydrogels, however it can be deduced that PT-I and PD-I have stronger network than PO-I. PD-I can be described as a strong gel ($\tan \delta_{\text{LVER}} = 0.099$; $A''/A' = 0.08$), and PO-I and PT-I are moderately strong gels ($\tan \delta_{\text{LVER}}$ are 0.163 and 0.117, respectively) (Table 2). PD-I has solid-like behaviour with a high degree of elasticity, while PO-I and PT-I also exhibit solid-like behaviour but can be more easily deformed than PD-I. Yu et al. (2017) have reported frequency independence for G' and $\tan \delta$ of KOH fraction (which corresponds to the fraction I), and described it as a gel. Even though Zhou et al. (2020) have used different extraction protocol (CP fraction was extracted at 25 °C, while HP fraction was extracted at 80 °C), their HP fraction was reported as a weak gel. They also reported lower value of frequency exponent (b') for HP (0.16) than for CP (1.31) which is consistent with the presented trends (PO-C>PO-H>PO-I) and indicates lower frequency dependence of the HP fraction (Zhou et al., 2020).

Table 1. Power law parameters (A, b) and R^2 (goodness of fit) of G' and G'' as measured by frequency sweep. Values are expressed as mean of three replicates $\pm$ standard deviation. Different letters represent statistically significant ($p < 0.05$) difference between samples, as determined by ANOVA followed by post-hoc Tukey test. Capital letters indicate significant difference between fraction (C, H, I) of the same species, and small letters indicate significant difference between same fraction of different species (PO, PT, PD).

	G'			G''				
	A' (Pa)	b'	R^2	A'' (Pa)	b''	R^2	A'' / A'	b' / b''
PO-C	0.20 ± 0.00 Cb	0.33 ± 0.12 Ab	0.915	0.72 ± 0.18 Bb	0.51 ± 0.08 Aa	0.991	3.60	0.65
PO-H	5.76 ± 0.29 Bb	0.28 ± 0.01 Aa	0.991	3.77 ± 0.16 Aa	0.21 ± 0.01 Bb	0.996	0.65	1.33
PO-I	25.20 ± 1.69 Aa	0.09 ± 0.00 Ba	0.971	4.22 ± 0.20 Aa	0.08 ± 0.00 Cc	0.961	0.17	1.13
PT-C	0.26 ± 0.02 Bb	0.56 ± 0.01 Aa	0.954	0.84 ± 0.02 Bab	0.57 ± 0.01 Aa	0.999	3.23	0.98
PT-H	11.39 ± 0.71 Aa	0.08 ± 0.00 Bb	0.976	2.18 ± 0.25 Ab	0.21 ± 0.02 Bb	0.979	0.19	0.38
PT-I	11.08 ± 0.38 Ab	0.03 ± 0.00 Cb	0.946	1.22 ± 0.12 Bc	0.11 ± 0.01 Cb	0.906	0.11	0.27
PD-C	0.49 ± 0.05 Ba	0.51 ± 0.05 Aa	0.977	1.01 ± 0.04 Ba	0.56 ± 0.01 Aa	0.996	2.06	0.91
PD-H	1.24 ± 0.11 Bc	0.29 ± 0.03 Ba	0.987	1.19 ± 0.08 Bc	0.41 ± 0.02 Ba	0.996	0.96	0.71
PD-I	23.57 ± 1.51 Aa	0.03 ± 0.00 Cb	0.924	1.94 ± 0.16 Ab	0.14 ± 0.01 Ca	0.957	0.08	0.21

The amplitude sweep is another important test that utilises changes in strain, while keeping the frequency constant, in order to assess the behaviour of the material under applied deformation. Amplitude sweep also gives us G' and G'' values, as well

as crossover point ($G'=G''$) which is a strain value where the material transitions from predominantly elastic to predominantly viscous behaviour (Hyun et al., 2011; Mezger, 2015). When expressing the shear stress value at which crossover point happened, expression “flow point” is used. Yield point is also an important parameter, which indicates the end of linear viscoelastic range and the start of structure breakdown (Mezger, 2015). Amplitude sweep results are presented in Fig. 6C and corresponding results in table 2. Fractions that are characterised as viscoelastic fluids were below detection limit (PO-C, PT-C, PD-C, and PD-H). PO-H had the lowest G' (10.23 Pa, Table 2), which agrees with the results from Zhou et al. (2020), whose 1% HP fraction had G' of ~ 11 Pa. It is apparent that PO-H and PO-I had the shortest linear viscoelastic range (Fig. 6C), meaning that its yield point, flow point, and crossover point appeared at a lower shear stress (τ) than in PT and PD fractions (Table 2). However, PO-I had the highest G' (29.22 Pa, as compared to $G'(\text{PT-I})=12.30$ Pa, and $G'(\text{PD-I})=24.73$ Pa), thus it can store the most energy, but it becomes weaker and its structure breaks down at lower deformation than PT-H, PT-I, and PD-I. This can also be deduced from FTI values (flow transition index) (Table 2). FTI is a ratio of flow point and yield point and informs about the yield zone and breakdown behaviour. The yield zone is a zone between yield point and flow point, where storage modulus of a material is still predominant over loss modulus, but structural strength has decreased (Hyun et al., 2011; Mezger, 2015). In Fig. 6C, yield zone is marked by stars. If FTI value is close to 1, this indicates that the material is prone to brittle fracturing. In this research, none of the gels are prone to brittle fracturing as FTI values are above 1 (PO-H has the lowest value – 4.53, and PT-I as the highest value – 24.89) (Table 2), which shows high flowability. However, PT-I and PD-I show extremely high FTI, thus they can sustain the most deformation before G'' overtakes G' , even when inner gel structure is compromised. FTI of 22.93 was reported for gellan gum treated with 10 mM CaCl_2 and 0.25 mM NaOH (Fan et al., 2022). FTI of gellan gum is comparable to FTI of PD-I, however the difference in concentration was two-fold (PD-I – 1%, gellan gum – 2%), thus showing the remarkable properties of PD-I. The G'' curve of PO-I and PD-I show similarities – they are stable until a certain point during deformation (for PO-I: $\dot{\gamma} \sim 1$ 1/s and for PD-I: $\dot{\gamma} \sim 0.3$ 1/s), after which the G'' rises, but the crossover point does not yet occur. This can be explained by the occurrence of some bonds in the gel network breaking with

microcracks developing, while the overall gel structure is still stable and G' is dominant (Mezger, 2015; Ross-Murphy, 1995; Ross-Murphy & Shatwell, 1993). However, with the strain increase, microcracks start growing, the inner network breaks down, and the gel becomes viscous. This behaviour is also known as weak strain overshoot (marked by arrow in Fig. 6C), and is reported for emulsions, suspensions, pastes, soft hydrogels, polymer solution systems, xanthan gum solution, and others (Hyun et al., 2011). For example, soy protein hydrolysates have shown strain overshoot caused by flexible and disordered aggregates that, at higher strain, could form entanglements and contribute to shear thickening behaviour and higher G'' (Xia et al., 2021). An early study of psyllium husk (polysaccharides extracted with 2.5M NaOH) rheology (Haque et al., 1993) described it as a “xanthan-like weak gel” which was explained by the association of supramolecular fibrils that form cohesive gels at higher concentrations during longer resting times. In PT-I, however, this is not the case. G'' curve is fairly uniform until the crossover point, suggesting that G'' is more consistent (i.e. dissipated energy is more consistent) throughout the applied strain range, further highlighting the differences of the Australian species studied here compared to commercial psyllium.

Table 2. Amplitude sweep parameters for viscoelastic gels. G' and G'' (Pa) are storage and loss moduli. $\tan \delta$ is calculated as G''/G' in linear viscoelastic range (LVER). Yield point (Pa) is shear stress (τ) point where the structure of the material begins to weaken. Crossover point represents $G'=G''$, i.e. point after which viscous modulus dominates over elastic modulus (Pa). Flow point (Pa) is expressed as shear stress (τ) point at which $G'=G''$. Flow transition index (FTI) is calculated as crossover point (τ)/yield point (τ). Values are expressed as mean of three replicates $\pm$ standard deviation, except $\tan \delta$ and FTI, which are calculated from pre-existing parameters.

	G' (Pa)	G'' (Pa)	$\tan \delta_{\text{LVER}}$	Yield point (τ_y , Pa)	Crossover point ($G'=G''$, Pa)	Flow point (τ_f , Pa)	Flow transition index (τ_f/τ_y)
PO-H	10.23 $\pm$ 0.85	6.23 $\pm$ 0.38	0.610	2.74 $\pm$ 0.24	4.96 $\pm$ 0.23	12.42 $\pm$ 2.66	4.53
PO-I	29.22 $\pm$ 1.92	4.77 $\pm$ 0.24	0.163	8.90 $\pm$ 0.41	10.82 $\pm$ 1.51	42.15 $\pm$ 3.83	4.74
PT-H	13.51 $\pm$ 0.68	3.18 $\pm$ 0.35	0.236	4.49 $\pm$ 0.79	3.71 $\pm$ 1.07	80.45 $\pm$ 3.62	12.39
PT-I	12.30 $\pm$ 1.22	1.44 $\pm$ 0.18	0.117	5.01 $\pm$ 0.32	2.30 $\pm$ 1.62	124.63 $\pm$ 22.06	24.89
PD-I	24.73 $\pm$ 1.77	2.44 $\pm$ 0.09	0.099	4.88 $\pm$ 1.33	6.89 $\pm$ 0.65	97.66 $\pm$ 7.76	20.02

Therefore, PO, PT, and PD show varying rheology profiles of their mucilage fractions, as well as fractionation profiles (Fig. 1C, discussed in chapter 3.1). PO shows a transition from viscoelastic fluid to moderately strong gel gradually (from C, the outermost layer, to I, the layer closest to the seed) while PT and PD have more abrupt changes in gel strength when comparing mucilage fractions: PT has two fractions that can be described as strong (I) and moderately strong gel (H), while fraction C is viscoelastic fluid. On the other hand, PD-C and PD-H are viscoelastic fluids, and PD-I is a strong gel. It is important to note that PD-I represents 75.67% of overall mucilage (Fig. 2C), meaning that majority of the mucilage is, in effect, a strong gel. Similarly, PT-H and PT-I constitute 87.96% of the PT mucilage, also indicating its high gelling capacity. A schematic representation of the rheology of mucilage layers is shown in Fig. 7, which represents mucilage layer of PO, PT, and PD seeds and their proportions, whereas the density of the dots represents the gel strength (the denser the pattern, the stronger the gel). This depiction helps visualise that PD mucilage as a whole is stronger than PO and PT, as its fraction I has the largest proportion. Moreover, fractions H+I in PT and PD represent the larger proportion of the mucilage than H+I in PO.

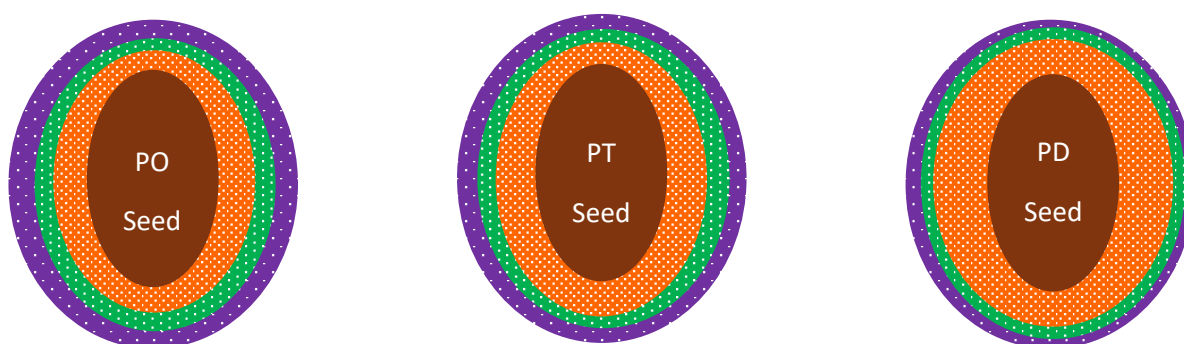


Figure 7. Schematic representation of mucilage fraction distribution and gel/fluid strength. Different colour represent mucilage layers: C – purple, H – green, I – orange. Density of dots represents gel strength (increased density – increased gel strength). Gel strength of all seeds was weaker in the outermost layer, and the strength increased with the mucilage closer to the seed. Note: mucilage and density of dots is not depicted to scale or to actual values.

To explain the behaviour and trends in rheology profile, correlation analysis of all studied properties was carried out (Fig. 8). Uncovering rheological behaviour in *P. ovata* is an ongoing topic of research, as its mucilage is not primarily pectic (Phan & Burton, 2018) and its heteroxylans do not behave as previously studied cereal arabinoxylans (Cowley & Burton, 2021). As mentioned, their extractability gets lower with further steps (such as alkaline extraction), and they have more densely branched side chains displaying complex interactions. The correlation analysis has shown significant positive correlation between yield and arabinose content ($r = 0.93$), but there was no significant correlation for any other monosaccharide. Moreover, yield was positively correlated with A+X and with A:X ratio ($r=0.75$ and 0.94 , both significant (Fig. 8). This points to highly branched fibre fraction being the most abundant polysaccharide. Yu et al. (2017), have suggested that the hydrogen bonds between the side chains, and sidechains and backbone are the primary driver of physical gel formation. Here, both the pectin component (expressed as rhamnose + galacturonic acid), pectin:arabinoxylan ratio, and A:X ratio seem to be the determinants of gelling behaviour of each mucilage layer in each species. Parameters b' and b'' (power law parameters which represent slope of G' and G'' as measured by frequency sweep) are positively correlated with: rhamnose ($b' = 0.69$, $b'' = 0.65$), galacturonic acid ($b' = 0.77$, $b'' = 0.72$), and pectin:arabinoxylan ratio ($b' = 0.69$, $b'' = 0.64$) (Fig. 8). This points to conclusion that presence of pectin has significant effect on flowability of mucilage fraction. For example, PD-C has shown the highest frequency dependence, which is characteristic for viscoelastic fluids ($b' = 0.51$, $b'' = 0.56$), and the highest rhamnose content of all fractions (15.468%). Additionally, the studies on PD seem to confirm this point – PD-C and PD-H have the same A:X ratio (0.11), but different pectin:arabinoxylan ratio (PD-C = 0.49, PD-H = 0.12). They also have different $\tan \delta$ values (PD-C~2, PD-H~1), which suggest the importance of the pectin component on viscoelastic behaviour. This agrees with extractability as well – fraction C is cold water extractable, which is due to the presence of pectin. Its negative charge is presumed to be responsible for increased solubility, while AX is less soluble. On the other hand, A:X ratio seems to affect the gel strength, as positive correlations

are reported for A:X ratio and yield point (0.74), crossover point (0.69), and flow point (0.69) (Fig. 8). This partly agrees with the mechanism of gelation previously proposed (Haque et al., 1993; Ren et al., 2020; Yu et al., 2017) – fraction C, which has more complex side chains and diverse composition forms viscoelastic fluid, since said complexity prevents requisite molecular associations that would result in a viscoelastic solid. Yu et al. (2017) have also highlighted importance of terminal arabinoses in fraction H, and xylose in side chains in fraction I as drivers of hydrogen bonds in those fractions, and consequent rheology profile (Yu et al., 2017, 2018, 2019, 2021). Moreover, overall arabinoxylan (A+X) showed positive correlations with G' , G'' , A' , A'' , CP, YP, FP, and FTI, indicating that arabinoxylan content was important for gel strength and resilience under deformation, in addition to the density of side chains (expressed as A:X ratio) (Fig. 8). It can be surmised that correlation analysis confirmed the proposed relationship between the fibre structure and the rheology profile. This relationship has been discussed previously for *P. ovata*, but not for *P. turrifera* or *P. drummondii* due to the lack of characterization. Further studies, such as exact molecular weight determination, intrinsic viscosity determination, linkage analysis, and temperature sweep could provide deeper insight into mechanisms and interactions of *Plantago* polymers, and help develop further hypotheses which would explain *P. turrifera* and *P. drummondii* properties. Nevertheless, with the information provided from this study, it can be concluded that pectin is the driver of extractability and viscous behaviour, while the content and the yield of the AX are important for gel strength and resilience.

		Yield	Relative yield	Protein	Mannose	Rhamnose	Glucuronic acid	Galacturonic acid	Glucose	Galactose	Xylose	Arabinose	Pectin:AX	A+X	A:X	A:X (FTIR)	G'	G''	tan delta	A'	b'	A''	b''	A''':A'	b''':b'	Yield point	Flow point	Crossover point	FTI
Yield		1.00																											
Relative yield		0.78	1.00																										
Protein		-0.59	-0.35	1.00																									
Mannose		-0.38	0.22	0.44	1.00																								
Rhamnose		-0.46	-0.50	0.00	0.08	1.00																							
Glucuronic acid		-0.49	-0.08	0.85	0.58	-0.17	1.00																						
Galacturonic acid		-0.42	-0.54	-0.06	-0.02	0.98	-0.22	1.00																					
Glucose		-0.41	-0.13	0.82	0.62	-0.14	0.66	-0.20	1.00																				
Galactose		-0.50	0.03	0.70	0.81	-0.19	0.88	-0.25	0.75	1.00																			
Xylose		-0.09	-0.37	-0.36	-0.54	-0.17	-0.44	-0.11	-0.48	-0.48	1.00																		
Arabinose		0.93	0.79	-0.44	-0.36	-0.65	-0.28	-0.64	-0.31	-0.32	-0.11	1.00																	
Pectin:AX		-0.42	-0.48	-0.02	0.05	0.99	-0.17	0.99	-0.14	-0.19	-0.23	-0.61	1.00																
A+X		0.75	0.45	-0.60	-0.64	-0.67	-0.51	-0.61	-0.57	-0.57	0.52	0.79	-0.67	1.00															
A:X		0.94	0.82	-0.39	-0.30	-0.60	-0.24	-0.58	-0.26	-0.28	-0.23	0.99	-0.55	0.71	1.00														
A:X (FTIR)		0.80	0.61	-0.50	-0.38	-0.58	-0.25	-0.49	-0.34	-0.24	-0.08	0.83	-0.50	0.67	0.83	1.00													
G'		0.78	0.63	-0.53	-0.34	-0.59	-0.34	-0.53	-0.33	-0.31	0.00	0.84	-0.52	0.72	0.82	0.89	1.00												
G''		0.45	0.32	-0.53	-0.33	-0.56	-0.35	-0.48	-0.32	-0.19	0.28	0.54	-0.49	0.64	0.46	0.63	0.74	1.00											
tan delta		0.27	0.17	-0.43	-0.28	-0.43	-0.33	-0.38	-0.27	-0.15	0.35	0.34	-0.39	0.51	0.26	0.34	0.43	0.90	1.00										
A'		0.81	0.65	-0.52	-0.34	-0.56	-0.34	-0.50	-0.33	-0.34	-0.04	0.85	-0.49	0.71	0.84	0.89	0.99	0.65	0.32	1.00									
b'		-0.27	-0.64	-0.30	-0.44	0.69	-0.54	0.77	-0.40	-0.53	0.39	-0.50	0.69	-0.19	-0.50	-0.27	-0.37	-0.15	-0.03	-0.37	1.00								
A''		0.45	0.20	-0.67	-0.50	-0.41	-0.52	-0.31	-0.47	-0.39	0.37	0.50	-0.34	0.66	0.42	0.67	0.78	0.94	0.76	0.71	0.05	1.00							
b''		-0.14	-0.55	-0.37	-0.51	0.65	-0.63	0.72	-0.47	-0.66	0.42	-0.38	0.64	-0.06	-0.39	-0.24	-0.34	-0.25	-0.15	-0.32	0.95	-0.03	1.00						
A'/A''		-0.24	-0.51	-0.20	-0.34	0.55	-0.41	0.59	-0.31	-0.39	0.33	-0.42	0.52	-0.16	-0.43	-0.24	-0.43	-0.34	-0.25	-0.42	0.86	-0.17	0.90	1.00					
b'/b''		-0.01	-0.38	-0.60	-0.57	0.18	-0.63	0.30	-0.53	-0.50	0.53	-0.09	0.23	0.24	-0.16	0.22	0.20	0.52	0.50	0.14	0.71	0.70	0.58	0.47	1.00				
Yield point		0.67	0.55	-0.51	-0.35	-0.57	-0.34	-0.53	-0.34	-0.31	0.07	0.78	-0.52	0.72	0.74	0.77	0.96	0.74	0.44	0.94	-0.37	0.79	-0.36	-0.44	0.23	1.00			
Flow point		0.65	0.51	-0.53	-0.33	-0.58	-0.30	-0.49	-0.31	-0.22	0.06	0.72	-0.50	0.66	0.69	0.88	0.97	0.83	0.53	0.94	-0.29	0.87	-0.32	-0.39	0.36	0.93	1.00		
Crossover point		0.65	0.51	-0.53	-0.33	-0.58	-0.30	-0.49	-0.31	-0.22	0.06	0.72	-0.50	0.66	0.69	0.88	0.97	0.83	0.53	0.94	-0.29	0.87	-0.32	-0.39	0.36	0.93	1.00	1.00	
FTI		0.84	0.72	-0.38	-0.31	-0.43	-0.38	-0.47	-0.30	-0.45	-0.08	0.86	-0.43	0.69	0.85	0.48	0.64	0.35	0.26	0.66	-0.41	0.29	-0.28	-0.40	-0.21	0.65	0.45	0.45	1.00

Figure 7. Correlation matrix (Pearson's correlation coefficients) for properties determined in this work. Negative values are coloured red, and positive values are coloured green. Bolded values are statistically significant at $p < 0.05$.

Overall, all gels show resistance to deformation. PO husk is used in food industry specifically for its gelling properties. It is the third most used addition in baking industry, and often it is used for gluten free breads (Belorio & Gómez, 2022). Rheology findings of PT and PD suggest that they have even higher gel strength, thus they could also be applied as gluten replacement. For example, they could be used in gluten-free whole grain bread (such as quinoa and buckwheat), hearth breads (which are baked directly on the oven floor or a baking stone, which demands more robust gluten network due to higher and more rapid temperature rise), or even in high fibre, gluten-containing breads, as all of them necessitate strong network formation. Moreover, PT and PD can be used food products which demand even higher structural integrity, specifically during production process, such as extrusion. This clearly shows potential of PT and PD in food industry and beyond.

4. Conclusion

This study explored fractionation behaviour, composition, and rheology of three distinct fractions from four *Plantago* species. *P. ovata*, commercially known as psyllium, and three relatives that grow in Australia, *P. lanceolata*, *P. turrifera*, and *P. drummondii* represent a range of hydration properties, which can be exploited in food product development. Monosaccharide analysis showed primarily heteroxylan composition, with varying amounts of pectin. Arabinose to xylose ratio increased with further extraction steps, indicating that the higher sidechain substitution leads to lower extractability and stronger gelling. Successful determination of molecular weight was proven to be dependent on the suitability of solvent and is planned for future work. This work represents first known monosaccharide composition analysis of *P. drummondii*, as well as first reported rheological studies of *P. turrifera* and *P. drummondii*. *P. lanceolata* did not form a gel at the used concentration (1%). A fraction from *P. drummondii* can be characterised as strong gel, while the other fractions are considered to be weak gels. With further extraction steps, gel strength and resilience were higher, showing that the alkaline extracted fractions were the most resilient against deformation. Rheology has uncovered potential of *P. turrifera* and *P. drummondii* as strong gels that could be utilised for multiple purposes in food industry. Even though *P. ovata* is known for its high viscosity and application in food products, this work clearly demonstrates the untapped potential of *P. turrifera* and *P. drummondii* mucilage as stronger gelling and structuring hydrocolloids.

5. References

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CHAPTER 4

Rheological characterization and enzymatic hydrolysis of composite
rice starch-*Plantago* fibre gels

Title:

Rheological characterization and enzymatic hydrolysis of composite rice starch-
Plantago fibre gels

Authors:

Lucija Strkalj^{1,2}, Gleb E. Yakubov², Rachel A. Burton¹, James M. Cowley^{1*}

¹School of Agriculture, Food and Wine, University of Adelaide, Waite Campus,
Urrbrae, SA, Australia

²Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group,
School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United
Kingdom

*Corresponding author

Highlights:

- *Plantago* fibre fractions significantly decreased freeze-thaw syneresis of rice starch.
- *Plantago* fibre fractions increased viscous rheological behaviour when added to rice starch.
- RS-fibre gels had lower yield point, higher flow point, and improved resilience.

Abstract:

Plantago fibres differ significantly in their chemistry and rheological properties that may be exploited to manipulate food quality. The impact of the addition of three fibre fractions from four *Plantago* species (*P. ovata*, *P. lanceolata*, *P. turrifera*, *P. drummondii*) on the properties of rice starch (RS) gels was studied. We fabricated twelve RS-fibre composite gels and measured freeze-thaw syneresis, frequency and amplitude sweep rheology, and starch hydrolysis by α -amylase. All fibres decreased the freeze-thaw syneresis significantly, which is desirable in food products

development. Frequency sweep revealed that the fibre addition increased viscous behaviour of RS, as $\tan \delta$ was increased in the majority of RS-fibre gels. Amplitude sweep revealed that all fibres, except *P. lanceolata*, increased G' and G'' , which could be the result of the gelling properties of *Plantago* fibre. Moreover, with the fibre addition, yield point was decreased, which points to disturbances in the RS gel network, but the flow point was increased, pointing to increased resilience under stress. This can be beneficial when developing starch-based food products with high texturizing stability. Starch hydrolysis did not show major differences in the hydrolysis extent, but the hydrolysis rate was significantly increased in all cases, except for a specific fraction from *P. ovata*. This was not correlated to the rheological properties, thus future studies should further probe that mechanisms that govern starch hydrolysis behaviour when fibre is added.

Keywords:

Plantago mucilage, rice starch-fibre composite gels, freeze-thaw syneresis, dynamic oscillatory rheology, starch hydrolysis

Abbreviations:

C = cold water fraction, H = hot water fraction, I = intense agitation fraction, PD = *Plantago drummondii*, PL = *Plantago lanceolata*, PO = *Plantago ovata*, PT = *Plantago turrifera*

1. Introduction

Rice is one of the most important worldwide crops, with the annual production of 520 million metric tonnes (U.S. Department of agriculture, 2023). It is the third most consumed cereal, after wheat and maize (Maraseni et al., 2018), and it is also one of the most utilised cereal base for gluten-free products (Park & Kim, 2023). However, its processing can cause decreased nutritional value and undesirable techno-functional properties, such as increased bread firmness, higher glycaemic index, and high cooking loss (Park & Kim, 2023). To overcome these undesirable occurrences when developing food products, oftentimes fibres or hydrocolloids are added (Zhou et al., 2022). One hydrocolloid of particular interest is the mucilage from *Plantago ovata* husk, also known as psyllium, a gel-forming heteroxylan with nutritional benefits on cardiovascular and gastrointestinal systems (Belorio & Gómez, 2022; Cowley & Burton, 2021; McRorie et al., 2021). *P. ovata* husk has been utilised as a source of added fibre in many products and added to different starches, including rice starch. Most often, the addition of *P. ovata* husk to rice starch-based products is studied in the context gluten free products. Its addition in rice flour gluten-free bread resulted in lower hardness and better scores in sensory testing (Hastaoğlu et al., 2024). When *P. ovata* husk and sugar beet fibre were added to gluten-free bread containing rice starch and corn starch, both fibres improved dough workability and techno-functional properties, but *P. ovata* husk displayed a significant anti-staling effect (Cappa et al., 2013). This can be explained by its high water absorbing capacity, which limits moisture loss during storage. Rice crackers with psyllium husk powder, but not without, were comparable to wheat crackers, as assessed by texture analysis and sound release, which is desirable (Ren et al., 2021). Noodles made from high-resistant starch rice flour with psyllium husk and psyllium powder had improved breakage tolerance, cooking loss, and chewiness, and decreased estimated glycaemic index (66.74 and 65.77, compared to RS only noodles with had 86.89 eGI) (Gong et al., 2024). The authors attributed these results to increase in starch crystallinity, as well as higher soluble fibre content of psyllium powder. Overall, *P. ovata* husk is reported to have positive effect on physico-chemical and storage properties, as well as digestibility of rice based products.

When assessing the addition of a fibre in a starch, it is important to understand how this addition influences the hydrothermal treatment of starch, i.e. gelatinisation, and the final gel structure. During gelatinisation, the water enters the starch granules under increased temperature (up to 100 °C) which causes the granules to swell, burst, and form a starch hydrogel. Swelling capacity or swelling power of a starch is its ability to absorb and retain water during hydrothermal treatment. The swelling power of rice starch used in this work is 2.55 g/g (He et al., 2020). It has been shown that pea and cereal starches exhibit less swelling than tubers starches (Li & Yeh, 2001). Most often, fibre or hydrocolloid addition prevents or reduces complete gelatinisation as they absorb the water, making it less available for starch (Chen et al., 2022). Water solubility index of starch is a measure that determines the amount of polysaccharides or soluble components released from starch granules when they are exposed to excess water. It is expressed as a percentage of the original weight of the sample that dissolves in water. The water solubility index of rice starch used in this work is 1.97 % (He et al., 2020). In general, rice starch has low solubility, as its solubility was reported lower than the potato, tapioca, wheat, and maize starch solubility (Mukerjee et al., 2007).

P. ovata is the most often used *Plantago* species studied in the context of improvement of products containing rice flour or rice starch. Considering that there is more than 200 *Plantago* species, with many of them mucilage producing and with varied properties, their potential is underexplored (Cowley et al., 2021; Cowley & Burton, 2021). Moreover, *P. ovata* husk produces mucilage which forms a strong gel, and it is reported that its fractions have varying gelling and rheological properties, opportunities for fine-tuning food product structure, texture, and quality by choosing a fraction with specific viscoelastic profile arise (Yu et al., 2017). Therefore, the aim of this work is to explore addition of three fractions from four different *Plantago* species, which are *P. ovata*, *P. lanceolata*, *P. turrifera*, and *P. drummondii*. In the previous chapter, the fibre fractions displayed distinct fractionation behaviour, related to their monosaccharide composition, and an in-depth rheological characterisation of these specific *Plantago* fractions was conducted. The fractions showed a range of viscoelastic behaviours, which corresponded closely to their hydration capacity and

monosaccharide composition. In Chapter 2, the potential strategies to expand *Plantago* mucilage use in food products were proposed, where two of the strategies was the use of fractions and the use of alternate *Plantago* species. Therefore, with the knowledge from the previous experiments, the impact of the fractions in a simple rice starch-fibre system is explored, which could illustrate potential utility of said fractions in starch based food products. We explored addition of 12 *Plantago* fractions to rice starch gel, and examined their appearance, freeze-thaw syneresis, amplitude and frequency sweep, and starch hydrolysis by α -amylase.

2. Materials & methods

2.1. Materials

Rice starch was purchased from Sigma Aldrich (S7260, Sigma Aldrich, Australia). Amylose content was 31.2%, as determined by 96-well plate iodine binding colorimetric assay (Kaufman et al., 2015)

Seeds of *P. ovata*, *P. lanceolata* and *P. turrifera* were originally sourced and produced as per Cowley et al. (2021), and where applicable, collection complied with relevant institutional, national, and international guidelines and legislation. Seeds of *P. drummondii* were originally collected on private land near Red Cliffs, Victoria, Australia.

Porcine pancreatic α -amylase (E-PANAA) was purchased from Megazyme (Neogen Australasia, Queensland). Amylose from potato (A0512), D-maltose monohydrate (63418), 4-hydroxybenzhydrazide (H9882), trichloroacetic acid (T0699), sodium carbonate (223530), iodine (207772), and sodium phosphate monobasic (S0751) were purchased from Sigma Aldrich (New South Wales). Sodium phosphate dibasic (SA026), potassium iodide (PA001), and sodium hydroxide (SA000) were purchased from ChemSupply (South Australia, Australia). Hydrochloric acid (RP1104) was purchased from RCI Labscan (Victoria, Australia). Mineral oil (330779) was purchased from Sigma Aldrich (New South Wales, Australia).

2.2. Methods

2.2.1. Sample preparation

Fibre fractions were extracted based on Yu et al.'s work (2017) and Cowley et al.'s work (2020) with some changes. Firstly, seeds were dispersed in 800 mL RO H₂O. Due to varying water absorption and gelling capacities, different amount of seeds were used for extraction to allow proper mixing: *P. ovata* – 20 g, *P. lanceolata* – 40 g, *P. turrifera* and *P. drummondii* – 8 g. Seeds were stirred with magnetic stirrer for 4 h at 25 °C, after which they were strained through a conventional kitchen strainer (c. 2 mm pore size), and strained fraction was dried (oven drying at 95 °C for 12 h), and ground with a coffee grinder (Sunbeam, Sydney, Australia) until the powder passed through 200 µm test sieve (Mesh 80, Haines Educational, Wantirna South, Australia). This fraction is named cold water fraction (C). The remaining gel and seeds (retained on the kitchen strainer) were re-dispersed in RO H₂O again (800 mL) and stirred at 65 °C for 4 h. The straining, drying, and sieving process was repeated, and this fraction is referred to as hot water fraction (H). Once again, the remaining gel and seeds (retained on the kitchen strainer) were extracted, this time by placing them in multiple 50 mL tubes, RO H₂O was added (approximate ratio of seeds+gel to water was 1:4), and the tubes were shaken (room temperature, 25 min, 1000 rpm) using a tissue-disruptor type mill (SPEX 2010 GenoGrinder, Australia). After shaking, the tubes were centrifuged for 45 min at 3600 rpm (5810R centrifuge, Eppendorf, New South Wales), and the gel layer was then carefully removed from the upper layer (H₂O) and seeds. The gel was dried overnight at 95 °C, ground using coffee grinder, and sieved through the 200 µm sieve as above. This fraction is intense agitation fraction (I).

The control gel (5% rice starch) was prepared by heating the rice starch in RO H₂O at 90 °C for 10 min. Rice starch-fibre gels are prepared by dispersing the fibre in RO H₂O (1%) at 90 °C for 30 min. Care was taken to re-add evaporated H₂O at the end of heating. After letting it cool to 25 °C, rice starch was added (5%, so the overall solids concentration was 6% w/v), and homogenised with homogeniser for 1 min at 1000 rpm to ensure rice starch was dispersed and there were no starch aggregates in the gel (ProScientific, USA). After homogenization, starch-fibre mixture was heated at 90

°C for 10 min. The gels were left to cool for 1 h before proceeding with experiments described below.

Each gel sample in the remainder of the text will be named as rice starch gel – RS, or rice starch with added fibre, with name of the species (*P. ovata* – PO, *P. lanceolata* – PL, *P. turrifera* – PT, *P. drummondii* – PD) and corresponding fraction (C, H, I). For example, RS-PD-H is rice starch with hot water fraction from *P. drummondii*.

For clarification, starch-fibre hydrogels generally can be made in three ways: 1) mixing and heating starch and fibre together, 2) solubilising fibre first and then adding the starch, followed by heating, and 3) heating the starch, adding the fibre and heating them together again (BeMiller, 2011). The first way is the most common, while the third is the least common. The second way is chosen in this work (solubilising and heating the fibre first, adding the starch, and then heating them together). The reasoning behind that is to ensure that the fibre fully gels, and that the matrix is viscosified. This would mean that there is less water available for starch gelatinisation (as the fibre absorbs the water) and it would affect the functionality of starch-fibre hydrogel. Since *Plantago* fibres are known for their high water uptake, it would allow for altered gelatinisation of the starch, as opposed to mixing and heating them together.

2.2.2. Appearance and colour measurement

A few grams of starch and each fibre in dried form was placed in square shaped weighing boats and photo was taken at constant distance with a digital camera (MIUI Global 14.0.11, China). Colour was determined with a handheld colorimeter CR-400 Chroma meter (Konica Minolta, South Australia), after calibrating with a white calibration plate. Similarly, photos and colour measurements were taken for RS and RS-fibre gels using the same method.

2.2.3. Freeze-thaw syneresis

Measurements of freeze-thaw syneresis of RS and RS-fibre gels was performed as described in the work of Ma et al. (2019) with minor changes. Samples were freeze-thawed for consecutive 15 days (22 h at -20 °C, followed by 2h at 40 °C), after which the upper water layer was carefully removed with Pasteur pipette without

centrifugation. Syneresis was calculated as a percentage of weight of gel after syneresis divided by weight of gel on day 0 using equation 1.

Equation 1:

$$\text{Syneresis (\%)} = \frac{\text{weight of gel after removing water layer (g)}}{\text{initail weight of the gel (g)}} * 100$$

2.2.4. Dynamic oscillatory rheology measurements

RS gel and RS-fibre gels, prepared as described in Section 2.2., were loaded onto the bottom geometry (smooth lower parallel plate, 35 mm diameter, TMP35/Ti, Haake Mars, Germany) of an air-bearing rheometer (Haake Mars iQ Air, Thermo Fisher, Germany). The upper smooth parallel plate (Haake Mars P35/Ti, Thermo Fisher, Germany) was lowered to a gap of 1.2 mm. After trimming of the edge of the gel with a plastic spatula and covering the edges with mineral oil to prevent evaporation during measurement, upper geometry was lowered to the measuring gap, which was 1 mm. Two dynamic oscillatory measurements were carried out: frequency sweep and amplitude sweep. Linear viscoelastic range (LVER) determination was firstly determined to ensure the measurements within the LVER. Temperature was kept constant at 25 °C by a heat exchanger (Haake HX-R, Thermo Fisher, Germany). Frequency sweep was measured from 62.83 rad/s to 0.6283 rad/s under constant deformation (shear strain 0.03). Amplitude sweep was measured under constant frequency of 6.283 rad/s to measure the crossover point. All measurements were carried out in triplicates. All data were analysed using RheoWin 4.91.0011. software.

Storage (G') and loss (G'') moduli of frequency sweep were fitted to a power law model using Equations 2 and 3:

Equation 2: $G' = A' * \omega^{b'}$

Equation 3: $G'' = A'' * \omega^{b''}$

G' and G'' are storage and loss moduli, ω is angular frequency, A' and A'' are intercepts at $\omega=1$ rad/s, b' and b'' are slopes. Coefficient of determination (R^2) is also reported.

2.2.5. Starch hydrolysis by α -amylase and determination of released reducing sugars by PAHBAH assay

Starch digestibility by α -amylase was determined according to Edwards et al. (2019). Starch to α -amylase ratio is kept constant in all samples, thus 100 mg of starch (in RS gel or in RS-fibre gel) was placed into 15 mL tubes and suspended in 10 mL phosphate buffered saline (pH=7.4). After briefly homogenising (PROScientific Premium MaX homogenizer, PRO Scientific Inc., CT, USA), the sample tubes were placed in a rotary mixer in an incubator (Bioline, Edwards Instrument Company Australia, New South Wales) at 37 °C and equilibrated for 30 min. Prior to reaction, 100 μ L of each sample was transferred into 1.5 mL microcentrifuge tube which contained 100 μ L of 0.3 M Na_2CO_3 ("stop solution"). The stop solution inactivates the enzyme and stops the reaction at each time point. To start the enzymatic reaction, porcine pancreatic α -amylase was added. Enzyme was carefully weighed before the start of the experiment, so the enzyme activity was 3 AU/mL, and dissolved in PBS. Preliminary experiments were carried out to determine the appropriate enzyme activity. One activity unit is defined as the amount of α -amylase needed to liberate 1.0 mg of maltose from starch in 3 min at pH=6.9 at 20 °C. Immediately after the addition of enzyme, tubes were returned into the incubator and rotated at 37 °C for the rest of the experiment. At pre-defined time points (2, 5, 7, 10, 15, 30, 90, 120, 150, 180 min) 100 μ L aliquots were taken from the reaction solution and placed into microcentrifuge tubes with an equal amount of stop solution. After brief vortexing, all tubes were centrifuged at 15 000 g for 5 min (Eppendorf centrifuge 5417R, New South Wales) to exclude any starch remains and 100 μ L of supernatant was placed into new microcentrifuge tubes, which were then used to determine the amount of reducing sugars.

The reducing sugar analysis (PAHBAH assay) was used to determine the amount of reducing sugars which are the product of α -amylase reaction at each sampled time point (Lever, 1972). Supernatants were diluted (1:20) in water, and 100 μ L of the diluted samples was placed in heat-safe microcentrifuge tube which contained 1 mL of PAHBAH reagent (preparation: 250 mg of *p*-hydroxybenzoic acid hydrazide dissolved in 4.75 mL of 0.5 M HCl and 45 mL of 0.5 M NaOH). The mixture is vortexed,

and then placed in boiling water bath (100 °C) for 5 min. After cooling to room temperature (15 min), 300 µL of each solution was transferred to a well in 96-well plate with flat bottom and the absorbance was measured at 405 nm (µQuant, Bio-tek instruments, Queensland). Standards containing known concentration of maltose (concentrations ranging from 100 µM to 1000 µM) were prepared the same way as described above and used for creating the calibration curve against which the samples were measured.

2.2.6. Statistical analysis

Data is presented as mean and standard deviation of at least triplicates. Starch hydrolysis curves were plotted in OriginPro 2022b software and fitted to first-order equation using non-linear regression, with parameter “a” as the extent of hydrolysis (C_{∞}) and parameter “b” as the rate of hydrolysis (constant k). Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey’s HSD post-hoc test ($p < 0.05$). Correlation analysis was determined by Pearson’s correlation ($p < 0.05$). Power law analysis for rheology frequency sweep data (G' and G'') were determined in GraphPad prism 9.0.0. (121). Graphs were made in GraphPad Prism 9.0.0. software, unless otherwise stated.

3. Results and discussion

3.1. Appearance and colour of fibres and rice starch-fibre gels

The successful implementation of novel additives will not only be determined by their direct functionality but also any unintended effects on organoleptic properties like colour. Fig. 1 shows the appearance and colour parameters of rice starch (RS) and fibres as powders (Fig. 1A), and RS-fibre gels (Fig. 1B). L^* indicates lightness. Parameter a^* represents the red/green colour coordinate, with the higher values representing red, and lower, green. Parameter b^* describes yellow/blue colour coordinate, where yellow has higher values, and blue has lower values. RS powder was the lightest in colour, with L^* value of 96.6, and correspondingly it produced the lightest starch gel ($L^* = 50.9$). It is noticeable that the fibres in powder form show a

wide range of beige and brown colours. Fraction C from PO, PT, and PD had the lightest colours (51.2, 52.3, 62.2, respectively), and corresponding gels were the most similar to the RS control (ΔE values were 7.7, 8.8, 9.7, respectively). All fractions from PL were very dark, with PL-I being the darkest of all fibres ($L^*=19.3$). Fractions I from all *Plantago* species were dark in colour, and resulted in brown RS-fibre gels. A possible reason for I fractions having darker colour than C or H fractions could be the proximity to the seed itself (as I is the innermost mucilage layer and the pigment layer is located just beneath the husk) (Phan & Burton, 2018), or there could be leaching of colour pigments from the seed itself during the intense agitation phase of extraction (section 2.1.). It has been shown that both *P. ovata* and *P. lanceolata* seeds are a source of phenolic compounds (Bahadori et al., 2020; Sagar et al., 2020) that may contribute to the colours seen here. In particular, RS-PL-I had a greyish hue, which would be especially undesirable for starch based food products which are generally susceptible to colour change due to their light colour. Interestingly, PO-I showed distinct orange hues, which made the fibre powder have a 'rusty' colour. PO-I had the highest a^* parameter (10.5), which corresponds to red colour. All fibre powders leaned towards yellow colour, rather than blue, however gels had lower b^* , suggesting a shift in the yellow hue, though this could be a result of a dilution effect when mixed with starch.

When comparing different concentrations of psyllium husk gel (1-7%) and thermal treatment (65 °C), it was reported that increased concentration resulted in higher L^* , a^* , and b^* parameters, while thermal treatment did not have a significant effect (Noguerol et al., 2022). It has been shown that 4% and 8% wheat flour substitution with psyllium husk or ground *P. ovata* seeds did not significantly change the L^* parameter of wheat bread, but the enriched bread had a slightly reddish colour, as reported by the authors (Pejcz et al., 2018). The colour change was explained by enzymatic oxidation of phenolic compounds by polyphenol oxidase. Overall, C and H fractions from PO, PT, and PD show the most commercial potential due to their light colour and modest effect on gel colour, while I fractions and PL mainly showed unacceptable colour changes in RS-fibre gels. However, there may be appropriate applications for these darker coloured fibres such as in applications where the matrix is naturally darker like a fibre enriched chocolate cake or rye bread.



Figure 1. Figure 1A shows colour parameters and images of fibre fractions. Figure 1B shows colour parameters and images of rice starch-fibre gels. Measurements are mean of three replicates $\pm$ standard deviation. Parameter L^* indicates lightness, a^* represents greenness to redness, and b^* represents blueness to yellowness. L^* , a^* and b^* of each sample are individually compared to the control rice starch by using Student's t-test and marked with asterisk (ns – not significantly different from the control, * - significantly different at level $p < 0.05$, ** - significantly different at level $p < 0.01$, *** - significantly different at level $p < 0.001$, **** - significantly different at level $p < 0.0001$). Differences between starch-fibre gels are analysed using one-way ANOVA with post-hoc Tukey test. Different letters note statistically significant difference at level $p < 0.05$.

3.2. Freeze-thaw syneresis of RS-fibre gels

Freeze-thaw syneresis is a measurement often used to 1) assess stability of starch-based products when exposed to low temperature and changes in environments, 2) study the effects of fibre or hydrocolloid addition in starch-gels, and 3) complement research experiments and understand the structure and interaction of starch and fibre. Fig. 2 shows freeze-thaw syneresis (after 15 days) of control RS gel and RS-fibre gels. All fibre fractions significantly lowered syneresis, meaning that they had a positive effect on the stability of RS gel, which showed 6.03% syneresis. The fibre type that had the most pronounced reduction on syneresis was PL-I, where the corresponding gel had 2.06% syneresis, however this was not significantly different from RS-I gels (Fig. 2). Even though they were not statistically significantly different, it appears that I Fractions lowered syneresis to a greater degree across the species, while Fractions C and H were similar and did not lower syneresis to the same degree as Fraction I. This trend is clear for PL, PT, and PD, whereas in PO mucilage the lowest syneresis was caused by PO-C. RS-PT-H had the highest syneresis of RS-fibre gels (4.13%), but was still significantly lower than the control RS gel.

There are multiple possible mechanisms to describe the effect of fibres in starch gels where syneresis is concerned. Dobosz et al. (2020) studied long-term (90 days) retrogradation of potato starch-xanthan gels reported that xanthan gum was able to bind water molecules and reduce their mobility, which reduced retrogradation and syneresis. However, the beneficial effect of xanthan gum depends on its concentration, and in which starch gel it is used, meaning that the amylose:amylopectin ratio of starch is an important determinant. In this case, syneresis results cannot be explained by water absorption properties of fibres alone. As mentioned above, RS-PL-I had the lowest syneresis (Fig. 2), but PL fibre had the lowest water absorption capacity (WAC = 39.81 g fibre/g water, Chapter 2, Fig. 1B). PT fibre had the highest WAC (121.95 g fibre/g water, chapter 2, Fig. 1B), but did not show the highest decrease in freeze-thaw syneresis. However, we measured WAC of seeds/total fibre, which cannot be directly comparable to freeze-thaw syneresis results. Moreover, other authors explain syneresis reduction by fibres via phase-separation of hydrocolloid-starch granule and hydrocolloid-amylose phases (Sae-kang

& Supphantharika, 2006). When konjac glucomannan was added to rice starch, it reduced syneresis and contributed to a more homogenous structure within the rice starch gel by inhibiting granule association (Charoenrein et al., 2011). In another study (Correa et al., 2013), locust bean gum (1 and 2%) had inconsistent effects on syneresis, where it was found that it depended greatly on the variety of rice and the concentration of locust bean gum. The higher concentrations that the authors tested (2%) seemed to increase the starch-starch interactions which would promote retrogradation and thus the water phase would be expelled more easily, leading to higher syneresis.

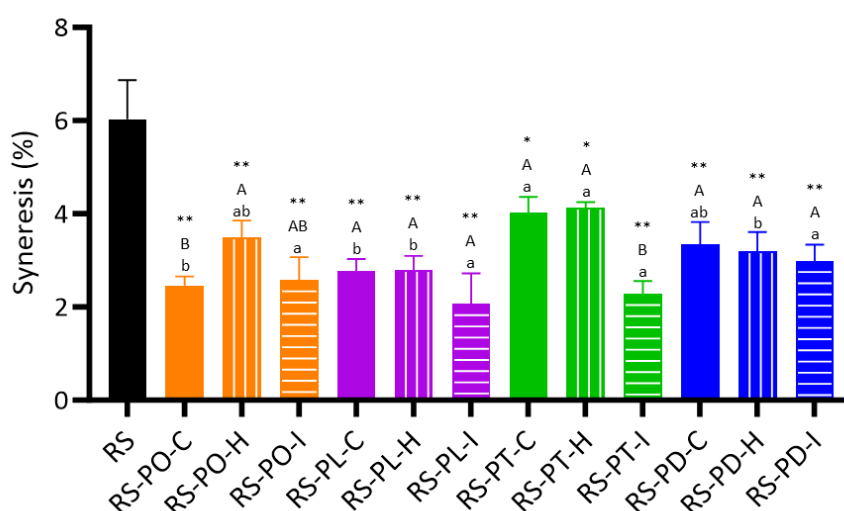


Figure 2. Freeze-thaw syneresis of RS-fibre gels. Measurements are means of 3 replicates. Freeze-thaw syneresis of each sample is compared to freeze-thaw syneresis of control RS gel by Student's T-test and marked with asterisk (* - $p < 0.05$, ** - $p < 0.01$). One-way ANOVA is applied to compare fractions of each *Plantago* species and statistically significant difference ($p < 0.05$) is marked with different capital letters. One-way ANOVA is also applied to compare same fraction (CWF, HWF or IAF) of all *Plantago* species and marked with small letters (different letters denote samples which are statistically significantly different, $p < 0.05$).

In general, all studied *Plantago* fibres could be used to decrease water separation in RS gels. Supplementary Fig. 1 shows superimposed syneresis of RS-fibre gels and WAC of each fibre fraction which was determined in the previous chapter. Pearson coefficient correlation was determined to be 0.6 (significantly positive) for syneresis

and WAC. However, this indicates that the fibre with higher WAC led to higher syneresis, which is opposite of the expected trend. In general, fibre with higher WAC leads to lower syneresis (Figueroa & Genovese, 2019). Therefore, the reduction in freeze-thawing cannot be explained by WAC of the fibre fractions. It seems that Fractions I decreased the syneresis the most. This might be related to the gelling capability of Fractions I, as rheology discussed in the previous chapter showed that Fractions I had the strongest gels. However, an exception is the PL fibre, which did not gel at all, had the lowest WAC, and had lower gelling capacity than Fractions C and H of all the other species, but it also lowered syneresis the most. The mechanisms behind the decrease in syneresis warrants further exploration, such as studying structure of each RS-fibre gel in frozen and thawed state by using different types of microscopy.

3.3. Rheology of RS-fibre gels

Due to the varied rheological properties observed in Chapter 2 (Section 3.5.) and the interesting syneresis results seen in this study, we applied dynamic oscillatory measurements to further characterise RS-fibre gels and probe the interactions between rice starch and *Plantago* fibres. Frequency sweep of RS-fibre gels is presented in Fig. 3 (viscoelastic moduli – Fig. 3A, $\tan \delta$ – Fig. 3B) while power law fitting parameters (A' , b' , A'' , b'') are in Table 1. When added to RS, all fibre except PL increased G' and G'' . All fractions of PL fibre decreased G' , with the most apparent decrease in RS-PL-H. RS-PL-H had A' parameter of 8.85 Pa (the lowest value of all samples, Table 1), while RS had A' of 26.72 Pa. Addition of PL fibres increased $\tan \delta$ values (Fig. 3B). It can be concluded that the addition of PL fibres decreased the amount of energy RS gel can store (due to decrease in G') and PL fibres changed the behaviour of RS gel towards viscous (as seen in the increase in $\tan \delta$ and A''/A'). As for the other fibres, PO-H and PT-H produced the highest A' (71.83 and 47.49 Pa, respectively) from PO and PT fractions. PD-I had the highest A' when compared to PD-C and PD-H (59.41, 30.58, and 51.69 Pa, respectively). Overall, G' was the highest in the RS-PO-H gel. $\tan \delta$ values were below 1, indicating weak gel behaviour in all of the studied gels (Fig. 3B). Of note is that the fibre addition changed $\tan \delta$: all C

fractions substantially increased $\tan \delta$, shifting the viscoelastic properties of the gel toward the viscous. One explanation for this is that C fractions are likely mostly soluble at the temperature used for rheometry as they were extracted at 25 °C, and therefore they would contribute more homogeneously to the intergranular liquid phase. A fraction from *P. ovata* extracted at 25 °C is characterised as viscoelastic fluid in our work (Chapter 2, Section 3.5) and in other research as well (Yu et al., 2017; Zhou et al., 2020). Moreover, parameter b' (representing frequency dependence) increased with the addition of C fraction (b' of RS gel was 0.08, and b' for RS-PO-C, RS-PT-C and RS-PD-C, were 0.18, 0.19 and 0.19, respectively). Similar findings are reported for other starch-fibre (or hydrocolloid) combinations. When HPMC or gum acacia were added (1, 1.5, 2%) to *Colocasia* starch (total solids content 8%), an increase in $\tan \delta$ was reported for both gums, at all concentrations (Shrivastava et al., 2018). The authors attributed this effect to depolymerization of gums at high temperature. We doubt this is the case for our samples, as thermogravimetric analysis for psyllium husk has shown that initial and final decomposition temperatures were 245 and 539 °C (Singh et al., 2007). Another type of behaviour can be seen in $\tan \delta$ graphs when C fractions and H fractions of PO, PT, and PD are added to RS, which is change in frequency dependence, depending whether the measurement is observed in lower or higher frequency (Fig. 3B). For example, the slope of RS-fibre at 1 rad/s are comparable to RS slope, but RS-fibre had lower slope than RS gel at 10 rad/s (Fig. 3B). An earlier study noted this behaviour when galactomannans (locust bean gum and guar gum) were added to waxy rice starch (Kulicke et al., 1996). This change in behaviour depending on frequency points to existence of composite gel network, as opposed to homogenous gel network. As they explained, galactomannans and waxy rice starch are thermodynamically incompatible with each other, and waxy rice starch forms permanent junction zones, while galactomannans form temporary entanglements (Kulicke et al., 1996). Therefore, with the presence of galactomannans and increasing concentration, composite gel behaviour became more viscous at the lower frequency, but at the higher frequency the higher amount of galactomannan is more resistant to flow (Kulicke et al., 1996). However, to confirm this theory as fully applicable in our system, a range of *Plantago* fibre concentrations would have to be tested, which is proposed as work for future directions.

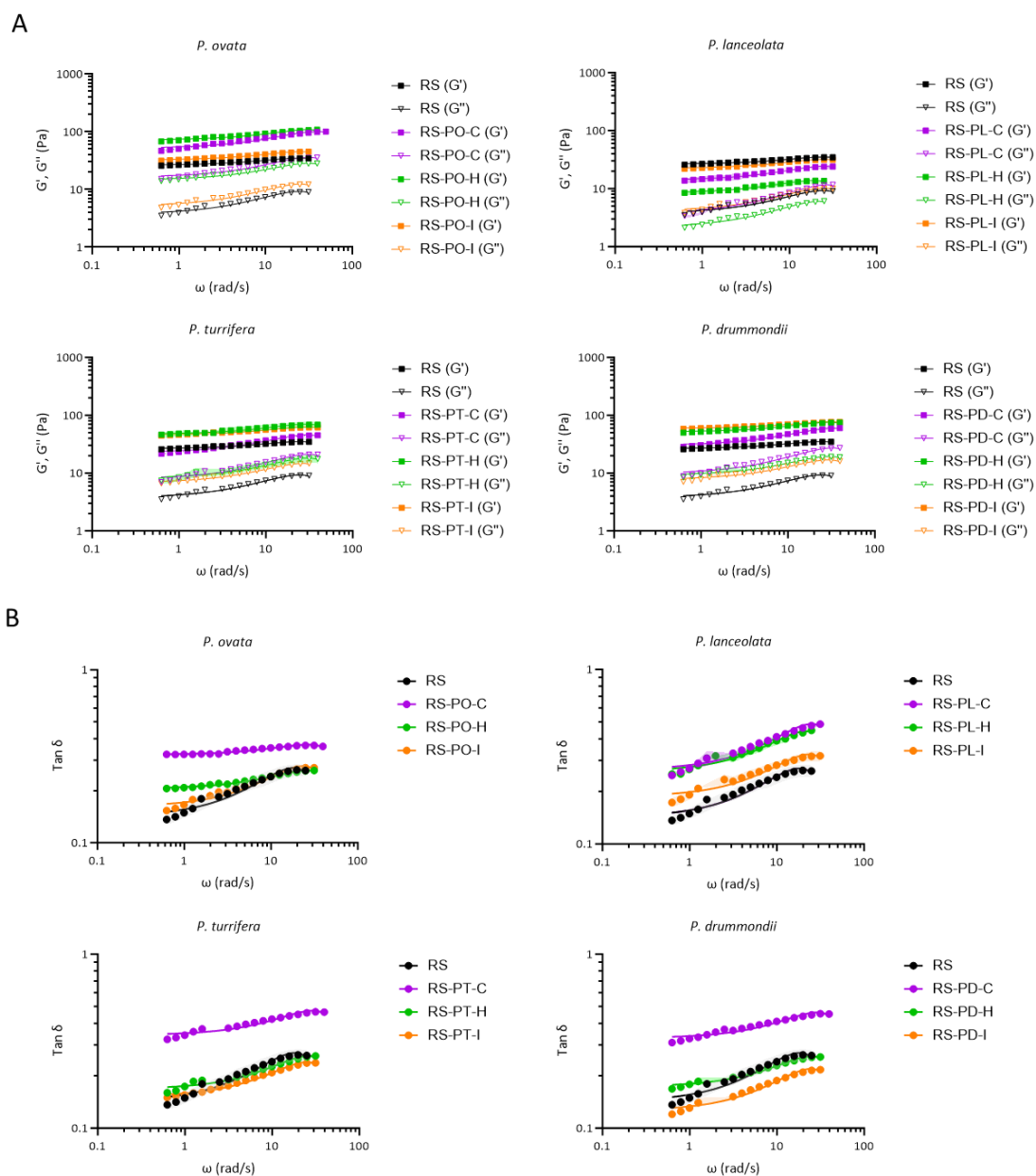


Figure 3. Frequency sweep of RS and RS-fibre gels. Figure 3A shows G' (full square) and G'' (empty inverted triangle), and figure 3B shows $\tan \delta$ (full circles) of three fractions (C, H, I) of all species. Each panel represents specific species, which is stated above the panel. RS gel is included on each panel (black colour) for ease of comparison between the control (RS) and RS-fibre gels. Each measurement is mean of three replicates and shaded parts represent standard deviation.

Table 1. Power law fitting parameters for G' (A' , b') and G'' (A'' , b'') of frequency sweep. Values are represented as mean of three replicates $\pm$ standard deviation. R^2 is goodness of fit. Parameters of each sample are individually compared to the control rice starch by using Student's t-test and marked with asterisk (ns – not significantly different from the control, * - significantly different at level $p < 0.05$, ** - significantly different at level $p < 0.01$, *** - significantly different at level $p < 0.001$, **** - significantly different at level $p < 0.0001$). Differences between starch-fibre gels are analysed using one-way ANOVA with post-hoc Tukey test. Capital letters indicate statistically significant difference between fractions (C, H, I) of the same sample, and small letters indicate difference between the same fraction from different *Plantago* species.

	G'			G''				
	A' (Pa)	b'	R ²	A'' (Pa)	b''	R ²	A''/A'	b'/b''
RS	26.72±3.43	0.08±0.01	0.9943	4.11±0.23	0.25±0.01	0.9865	0.15	0.32
RS-PO-C	51.59±0.86 *** Ba	0.18±0.00 **** Ab	0.9971	16.65±0.39 **** Aa	0.22±0.00 ** Bd	0.9967	0.32	0.82
RS-PO-H	71.83±4.55 *** Aa	0.12±0.00 *** Bb	0.9971	14.87±0.86 **** Ba	0.18±0.01 *** Cc	0.9921	0.21	0.67
RS-PO-I	32.69±1.86 ns Cc	0.10±0.00 ** Ca	0.9950	5.56±0.16 *** Cc	0.24±0.01 ns Aa	0.9905	0.17	0.42
RS-PL-C	14.44±1.60 ** Bd	0.16±0.00 **** Ac	0.9872	4.11±0.27 ns Ad	0.31±0.00 **** Aa	0.9887	0.28	0.52
RS-PL-H	8.85±0.87 *** Cc	0.14±0.00 **** Ba	0.9733	2.43±0.11 *** Bc	0.29±0.01 ** Ba	0.9917	0.27	0.48
RS-PL-I	22.72±1.43 ns Ad	0.10±0.01 * Ca	0.9852	4.51±0.07 * Ad	0.25±0.01 ns Ca	0.9865	0.20	0.40
RS-PT-C	23.32±0.79 ns Bc	0.19±0.01 **** Aa	0.9909	8.30±0.12 *** Ac	0.27±0.00 ** Ac	0.9890	0.36	0.70
RS-PT-H	47.69±4.99 ** Ab	0.11±0.01 ** Bb	0.9773	8.46±1.13 ** Ab	0.21±0.00 ** Bb	0.9773	0.18	0.52
RS-PT-I	45.94±1.96 ** Ab	0.09±0.00 ns Cb	0.9913	7.06±0.43 *** Ab	0.22±0.00 ** Bb	0.9922	0.15	0.41
RS-PD-C	30.58±0.37 ns Cd	0.19±0.00 **** Aab	0.9980	10.14±0.03 **** Ab	0.28±0.00 ** Ab	0.9957	0.33	0.68
RS-PD-H	51.69±3.55 *** Bb	0.11±0.01 ** Bb	0.9873	9.46±0.24 **** Bb	0.20±0.01 ** Bbc	0.9667	0.18	0.55
RS-PD-I	59.41±0.62 **** Aa	0.07±0.00 * Cc	0.9828	7.99±0.35 **** Ca	0.21±0.01 ** Bb	0.9744	0.13	0.33

After determining viscoelastic behaviour of the gels by frequency sweep, we utilised amplitude sweep to assess the behaviour of the gel in the linear viscoelastic range as well as under deformation, as that allows to measure the resilience of the gel. Therefore, we calculated yielding point (YP), flow point (FP), crossover point (CP), and flow transition index (FTI). Amplitude sweep graphs are presented in Fig. 4, and the corresponding parameters in Table 2. $\tan \delta_{\text{LVER}}$ seems to increase with the addition of C fraction of all *Plantago* species, pointing to the theory of viscous nature of the C fractions being the driver of the viscosity increase (G'' increase) in RS-fibre gels. The lowest $\tan \delta_{\text{LVER}}$ is determined for RS-PD-I, which was 0.168, indicating that it is a moderately strong gel. Similar to trends seen in the frequency sweep, all fibres except

PL (all fractions) increased G' when added to RS gel. Increases in G' can be a consequence of increases in concentration, however it is not a sole reason for this trend, as otherwise the increase would be the same in all the gels (including RS-PL gels) as the solids concentration of all gels is equal (1% fibre, 5% starch w/v). As mentioned, PL addition decreased G' , but it also decreased the crossover point (CP), yielding point (YP), and flow point (FP) (Table 2). This points to a disturbance of RS gel network structure by the presence of PL fibre. Moreover, PL fibre did not exhibit viscoelastic properties at 1% concentration (as detailed in Chapter 2, Section 3.5), therefore this disturbance could not have been 'compensated' by the elasticity of PL. Following this logic, one could speculate that increases in G' , G'' , CP, YP, and FP (Table 2) seen when PO, PT, and PD fibre fractions were added to RS is a result of the viscoelastic properties of the fibres themselves, but again this is most likely not the entire explanation. For example, G' of RS-PO-H is the highest of all gels ($G'=90.47$ Pa, which is 180% increase when compared to RS gel), but G' of 1% PO-H is lower than G' of other fibre fractions which were characterised as viscoelastic solids (for example, $G'(\text{PO-H})=10.23$ vs $G'(\text{PD-I})=24.73$ Pa, Chapter 2, Table 2). The addition of fibre fractions decreased the yield point (except RS-PO-C) and increased the flow point (FP of RS gel was 35.99 Pa, and the highest FP was 184.13 Pa which was reported for RS-PT-H) (Table 2). These trends of YP and FP indicate that the fibre addition shortened the linear viscoelastic range, which means lower predictability and resilience of a material (since LVER is a range in which G' and G'' act linearly, therefore we can predict material's behaviour), but higher FP means that a gel has higher resistance toward flow, i.e. it can withstand higher deformation (shear stress) until it starts to flow. This points that fibre addition disturbed rice starch network (therefore, lower YP), but the fibres' own resistance to flow increased FP. Again, this is a trend noticed for all fibres (except PL), but the fibre that has the highest FP on its own (1% PT-I - 124.63 Pa, Chapter 2, Table 2) did not result in the highest FP when added to starch ($\text{FP}(\text{RS-PD-I})=62.15$ Pa, vs $\text{FP}(\text{RS-PT-H})=184.13$ Pa). Further insight into the differences between each fraction when added to RS, could perhaps be given by determining molecular shape of the fibres, as it was shown that the rod-like shape of xanthan gum was critical to the shear-thinning properties of rice starch-xanthan mixtures (Kim & Yoo, 2006). Therefore, further studies into molecular weight and shape are recommended for

future research. Another important occurrence in starch gels is retrogradation. In our work, gels were allowed to cool down to 25 °C before they were measured on the rheometer, which could have allowed short-term retrogradation to occur (Dobosz et al., 2020). Short-term retrogradation is primarily driven by amylose-amylose interactions and the formation of continuous amylose network into which starch ghost and amylopectin are embedded (BeMiller, 2011). The presence of fibres could have disrupted amylose-amylose interactions, leading to a slower initiation of retrogradation, disrupting the amylose network and ultimately leading to lower YP. FTI parameter was increased with addition of the fibre, in all cases (Table 2). RS-PT-I had the highest FTI of 30.71. This parameter also confirms that the fibre addition increased gel resilience when the network is under deformation and is no longer in the linear viscoelastic range.

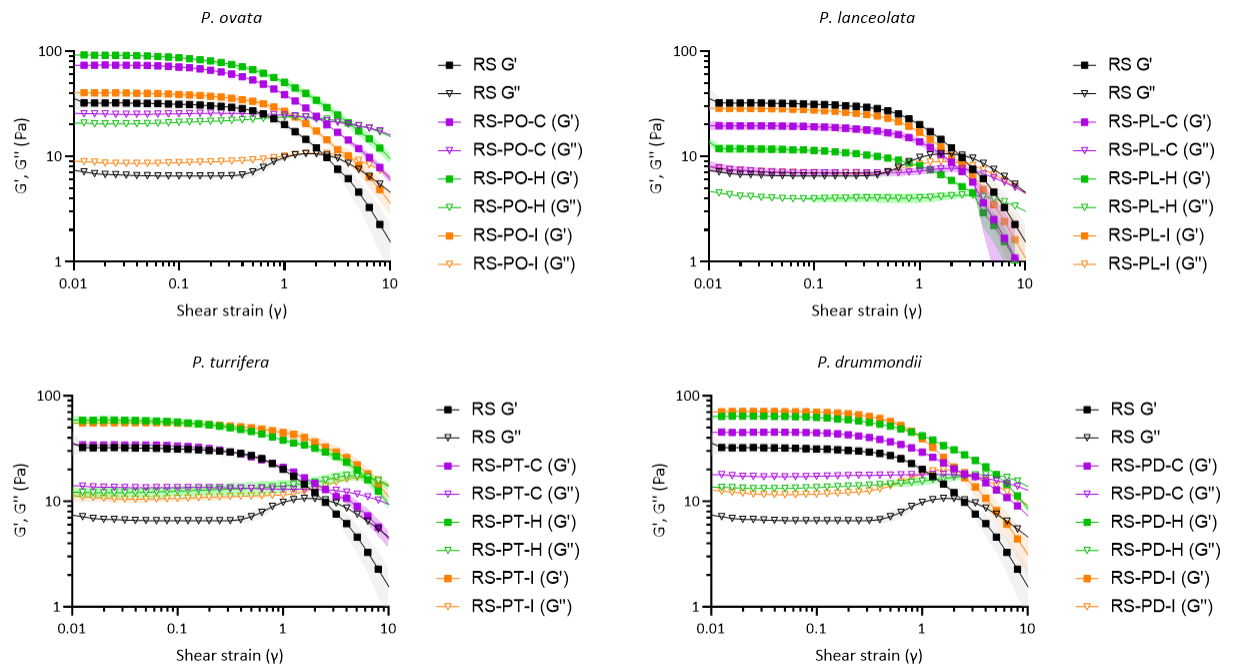


Figure 4. Amplitude sweep of all gels. G' is marked by full triangles, and G'' is marked by empty inverted triangles. Three fractions (C, H, I) of all species are shown. Each panel represents specific species, which is stated above the panel. RS gel is included on each panel (black colour) for ease of comparison between the control (RS) and RS-

fibre gels. Each measurement is mean of three replicates and shaded parts represent standard deviation.

Table 2. Amplitude sweep parameters of RS and RS-fibre gels (CP – crossover point, YP – yielding point, FP – flow point, FTI – flow transition index). Values are represented as mean of three replicates $\pm$ standard deviation. Parameters of each sample are individually compared to the control rice starch by using Student's t-test and marked with asterisk (ns – not significantly different from the control, * - significantly different at level $p < 0.05$, ** - significantly different at level $p < 0.01$, *** - significantly different at level $p < 0.001$, **** - significantly different at level $p < 0.0001$). Differences between starch-fibre gels are analysed using one-way ANOVA with post-hoc Tukey test. Capital letters indicate statistically significant difference between fractions (C, H, I) of the same sample, and small letters indicate difference between the same fraction from different *Plantago* species.

	G' (Pa)	$\Delta G'$ (%)	G'' (Pa)	$\Delta G''$ (%)	$\tan \delta_{\text{LVER}}$	CP	YP	FP	FTI
RS	31.66±3.63	/	6.67±0.30	/	0.212	10.43±0.77	17.84±0.28	35.99±2.32	2.02±0.14
RS-PO-C	72.99±0.62 ****	130.51	25.36±0.34 ****	280.15	0.347 ***	22.46±0.40 ****	18.94±0.38 *	69.21±5.94 ***	3.65±0.24 ***
	Ba		Aa		Ac	Aa	Aa	Ba	Cc
RS-PO-H	90.47±5.40 ****	185.74	20.58±0.78 ****	208.51	0.228 ns	20.76±0.97 ***	4.98±0.55 ****	113.00±13.71 ***	22.91±4.43 **
	Aa		Ba		Bb	Ba	Ba	Ab	Aab
RS-PO-I	39.69±2.47 *	25.34	8.73±0.16 ***	30.85	0.220 Ns	9.68±0.17 ns	5.14±0.61 ****	60.73±8.56 **	11.97±2.57 **
	Cc		Cb		Bb	Cc	Ba	Bb	Bb
RS-PL-C	18.00±1.19 **	-43.15	7.20±0.59 ns	7.86	0.379±0.01 ***	7.71±0.61 **	2.78±0.15 ****	25.58±2.14 **	9.25±1.21 ***
	Bd		Ad		Ab	Bd	Ad	Bc	Ab
RS-PL-H	11.64±1.09 ***	-63.23	4.09±0.20 ***	-38.70	0.352±0.02 ***	4.22±0.11 ***	1.81±0.45 ****	20.17±0.63 ***	11.56±2.69 **
	Cc		Bc		Aa	Cc	Bb	Ac	Ab
RS-PL-I	27.98±1.61 ns	-11.63	7.24±0.04 *	8.49	0.259±0.02 *	8.78±0.30 *	2.85±0.19 ****	30.36±1.24 *	10.67±0.40 ****
	Ad		Ac		Ba	Ac	Ab	Ac	Ab
RS-PT-C	34.05±0.88 ns	7.53	13.60±0.16 ****	103.88	0.400±0.01 ****	12.89±0.27 **	3.70±0.25 ****	45.83±1.12 **	12.43±0.65 ****
	Bc		Ac		Aa	Bc	Bc	Bb	Ba
RS-PT-H	58.41±5.32 **	84.49	12.15±1.35 **	82.18	0.208 ns	17.06±1.63 **	6.31±1.48 ***	184.13±18.45 ***	30.54±8.83 **
	Ab		ABb		Bb	Ab	Aa	Aa	Aa
RS-PT-I	55.45±2.81 ***	75.13	10.70±0.87 **	60.44	0.193±0.01 ns	16.25±0.62 ***	5.39±0.49 ****	164.40±21.36 ***	30.71±4.98 ***
	Ab		Ba		Cc	Ab	ABa	Aa	Aa
RS-PD-C	45.01±0.76 **	42.16	17.32±0.16 ****	159.64	0.385 ****	17.22±0.76 ***	5.92±0.09 ****	76.31±2.34 ****	12.90±0.20 ****
	Cb		Ab		Aab	Ab	Ab	Ba	Ba
RS-PD-H	63.64±3.80 ***	101.00	13.37±0.58 ****	100.44	0.210 ns	17.63±1.18 ***	5.89±0.04 ****	130.60±8.32 ****	22.17±1.32 ****
	Bb		Bb		Bb	Ab	Aa	Ab	Aab
RS-PD-I	70.57±1.92 ****	122.89	11.88±0.37 ****	78.13	0.168 *	19.28±1.30 ***	6.68±1.33 ***	62.15±2.93 ***	9.49±1.37 ***
	Aa		Ca		Cc	Aa	Aa	Cb	Cb

To the best of our knowledge, this is the first work reporting rheological characterization of rice starch gels with *P. ovata*, *P. lanceolata*, *P. turrifera* or *P. drummondii* fibres. Nonetheless, the impact on the addition of different types of fibre/hydrocolloids to rice starch or the impact of the addition of psyllium husk to other starch types could provide some valuable insight to the results seen here. In one study, corn starch was replaced by different amounts of psyllium husk (0, 2, 5, 10%) (Belorio et al., 2020). Psyllium replacement caused increase in G'' , but no changes in G' , irrespective of concentration. This is unlike our study, where both G' and G'' were increased. Methodology differences could be the key: while we added fibre to RS, Belorio et al. (2020) replaced the starch with fibre, meaning that it is difficult to distinguish between the effect of fibre addition and the effect of starch

reduction. They proposed a theory of rheological factors depending on free water availability, which explains their lack of changes in G' and increase in G'' , however this does not explain our results. For example, PT has the highest water absorbing capacity (Chapter 2, Fig. 1B), but RS-PT gels do not have the highest G' (table 2). When assessing the effect of xanthan gum, HPMC (hydroxypropyl methylcellulose), and guar gum in starch (overall concentration was 8%, starch was replaced at levels 0.2, 0.5, and 0.8%), Rossell et al. (2011) have reported that addition of hydrocolloids, rather than the reduction of starch had the primary effect on rheology. Starch dilution did decrease G' and G'' , but did not change $\tan \delta$, indicating that varying concentration did not change viscoelastic behaviour. On the other hand, hydrocolloids changed G' , but in specific ways: guar and xanthan gum increased G' and G'' with increased concentration, but HPMC did not show concentration-dependent behaviour. Similar to this research, hydrocolloid addition increased $\tan \delta$, where starch network shifted from elastic-like to viscous-like (Rosell et al., 2011). Further, their A' and b' parameters are also comparable to ours: both increased when fibre/hydrocolloid were included. They explained these trends as starch-hydrocolloid gels having “weak network structure where crosslinked zones alternate with ordered chain segments that do not allow crosslinking” (Rosell et al., 2011).

Overall, the addition of *Plantago* fibres to RS resulted in significant changes to the viscoelastic profile, depending on fibre fractions. These changes could be exploited for specific purposes. For example, food for people who have difficulties with swallowing is usually preferred to have $1 > \tan \delta > 0.1$, which includes all gels tested here, but specifically RS-PD-I, whose $\tan \delta$ is the lowest, and has the most prominent elastic part, which is safer for people with dysphagia (Wang et al., 2023). For products where food manufacturers would want to increase fibre content, but retain rheological properties of the original starch-based products, then addition of PL fibres may be the best option. Specifically, RS-PL-I displayed the least changed viscoelastic profile from the control RS gel. On the other hand, if developing a product which would need to be resistant to flow (i.e. have high flow point), then addition of PO, PT and PD fibre would be the better choice.

3.4. Starch hydrolysis by α -amylase of RS gel and RS-fibre gels

Rice is one of major crops in the world, source of starch, and important flour for gluten-free products. Improvement of rice starch and rice flour digestibility (i.e. decrease) has been an important subject of research (Qadir & Wani, 2022). It has been shown that brown rice had lower glycaemic potency than white rice due to its resistance to breakdown and prolonged rate of gastric emptying (Wu et al., 2017). Low acyl gellan gum decreased glycaemic index of white rice by coating and infiltrating rice grains during thermal treatment (Alshammari et al., 2024). Therefore, the effect of *Plantago* fibre fractions on rice starch hydrolysis by porcine pancreatic α -amylase was studied. Fig. 5 presents starch hydrolysis results: Fig. 5A shows hydrolysis curves, Fig. 5B and 5C show hydrolysis extent and rate with statistical analysis.

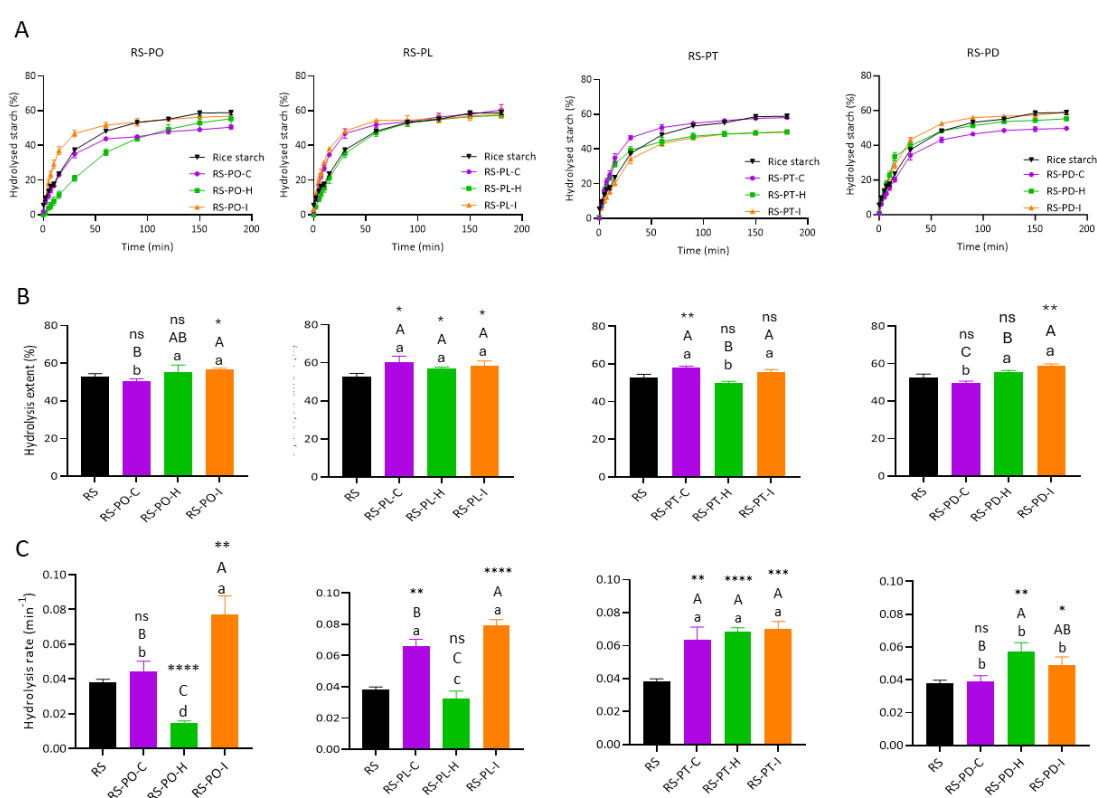


Figure 5. Starch hydrolysis of RS and RS-fibre gels by α -amylase. Figure 5A: starch hydrolysis curves sorted by each species, with the RS-species abbreviation written at the top of the panel. All fractions are represented by the same colour: C = purple, H = green, I = orange. RS control is included at every panel (black colour) for ease of

comparison. Figure 5B and 5C show hydrolysis extent (%) and hydrolysis rate (min^{-1}) of rice starch and rice starch-fibre gels. Hydrolysis extent and rate of each sample are individually compared to the control rice starch by using Student's t-test and marked with asterisk (ns – not significantly different from the control, * - significantly different at level $p < 0.05$, ** - significantly different at level $p < 0.01$, *** - significantly different at level $p < 0.001$, **** - significantly different at level $p < 0.0001$). Differences between starch-fibre gels are analysed using one-way ANOVA with post-hoc Tukey test. Capital letters indicate statistically significant difference between fractions (C, H, I) of the same sample, and small letters indicate difference between the same fraction from different *Plantago* species.

RS showed 52.75% hydrolysis, with the rate of 0.038 min^{-1} . The hydrolysis extent of RS measured here was lower than compared to some published works. For example, annealed RS (23% amylose) showed hydrolysis extent 30.6-50.3%, depending on the temperature of hydro-thermal treatment (Dias et al., 2010). The higher temperature changed the amorphous regions of the granule and appearance of surface pores, which could have promoted hydrolysis susceptibility (Dias et al., 2010). Another study focussed on different heat-moisture treatments (heating at 110°C for 4 h, at moisture content 10, 20, or 30%) and found that the rapidly digested starch (RDS) content was 84.0, 75.2, and 75.0% for 10, 20, and 30% HMT (Wang et al., 2018). Our RS gel was heated at 95°C for 10 min in 95% w/w H_2O , after which it was let cool to 25°C . This process allowed for the RS gel to form, which most likely included short term retrogradation (Dobosz et al., 2020). RS gel had G' of 31.66 Pa and $\tan \delta$ of 0.212 (Table 2), whose relatively strong structure could have caused lowered the potential digestibility.

The addition of *Plantago* fibre fractions impacted RS hydrolysis behaviour (Fig. 5), with the hydrolysis rate showing more significant changes than the hydrolysis extent. Fig. 5B shows that RS-PO-I, RS-PT-C, RS-PD-I, and all RS-PL gels had a higher extent of hydrolysis than the control RS gel, while other RS-fibre gels did not differ significantly from RS. The highest hydrolysis extent was measured in RS-PL-C (60.46%), followed by RS-PD-I (59.04%). The hydrolysis rate was increased in almost all samples, except in RS-PO-H, which decreased the hydrolysis rate by 60.5% (RS – 0.038 , RS-PO-H –

0.015 min⁻¹) (Fig. 5C). The only other gel where the hydrolysis rate was decreased was RS-PL-H (0.032 min⁻¹), but the decrease was not statistically significant. The highest hydrolysis rate was measured in RS-PL-I, followed by RS-PT-I (0.079 and 0.070 min⁻¹, respectively). Overall, it seems that *Plantago* fibres, except PO-H, increased the digestibility of RS. Specifically, significant increase in the hydrolysis rate could lead to increased glycaemic index *in vivo*, which is not beneficial for health (Goñi et al., 1997; Ren et al., 2016). In literature, the addition of fibres or hydrocolloids has mostly been reported as positive, meaning that the rice starch digestibility was decreased. When xanthan gum and locust bean gum (0.1, 0.3, 0.5 g/100 mL H₂O) were added to rice starch, both fibres decreased rapidly digested starch (RDS, digested within the first 20 min) and hydrolysis extent, and increased slowly digested starch (SDS, digested from 20 min to 120 min) and resistant starch (RS, undigested by 120 min) (Englyst et al., 1992; Zhang et al., 2023). This finding could be explained by the high solubility of these hydrocolloids, which increased viscosity of the starch-hydrocolloid mixture and coating of the granule surface, effectively hindering the access of α -amylase (Zhang et al., 2023). Interestingly, the authors found that there was no difference between 0.3 and 0.5 g/100 mL H₂O hydrocolloid addition, which they explained as a saturation point at which hydrocolloid interacts and envelops the starch granule, and when the point is exceeded, additional protective effects were not observed (Zhang et al., 2023). Pullulan addition (0.01-0.05%) decreased RDS and increased SDS+RS of rice starch, with changes being proportional to the amount of added pullulan (L. Chen et al., 2017). This result was explained as pullulan decreasing the amount of leached amylose and the formation of pullulan/amylose aggregates, with both mechanisms contributing to decrease of RDS. Moreover, pullulan decreased the rate and the extent of rice starch hydrolysis by α -amylase, which was attributed to pullulan-pullulan and pullulan-amylose structures, which could have coated the starch granule, and effectively blocked the access of the enzyme (Chen et al., 2017). Overall, it seems that the reported fibre and hydrocolloid addition to rice starch, rice flour, and rice grain decreases digestibility (Qadir & Wani, 2022). Therefore, one must ask the question: why do *Plantago* fibres seem to increase digestibility? Firstly, the hydrolysis extent is less affected than the hydrolysis rate (Fig. 5B and 5C), which indicates that the *Plantago* fibre addition did not change the overall amount of starch that was

gelatinised and hydrolysed. Correlation analysis has shown that the only significant correlation between gel properties and hydrolysis kinetics was between the hydrolysis extent and yielding point ($r=-0.58$) (Supplementary Table 1). This is a sensible finding, as a higher yield point indicates a stronger gel structure, which may result in lower digestibility. Secondly, even though fibre had limited effects on one aspect of digestibility (hydrolysis extent), it significantly increased the hydrolysis rate (Fig. 5C), which indicates that the fibre addition might have caused such changes to gelatinised starch matrix which would allow for easier enzymatic hydrolysis. Here, we present two possible scenarios, both leading to the same outcome. Considering there are no correlations between the rheology parameters and starch hydrolysis, we assume that the contrasting changes in RS viscoelasticity and, consequently, structure (decreasing and increasing viscoelastic moduli until a certain point) lead to a higher hydrolysis rate. To illustrate this, the highest hydrolysis rate was measured for RS-PL-I and RS-PT-I (0.079 and 0.070 min^{-1} , respectively), yet they both had opposing impacts on RS structure – PL-I slightly decreased G' and G'' , while PT-I significantly increased G' and G'' . *P. lanceolata* has a thin, non-robust layer of mucilage which does not form a self-standing gel at 1% w/v (Chapter 2, section 3.5), therefore it is not surprising that PL mucilage fractions decreased G' and G'' of RS, making the gel weaker and less resilient, which would enable easier diffusion of α -amylase throughout the matrix. On the other hand, *P. turrifera*, *P. drummondii*, and *P. ovata* significantly increased G' and G'' . As shown in Chapter 2, Section 3.5, these fibre fractions have differences among each other. Fraction C from all species were viscoelastic fluid, while Fractions H (except PD-H) and I were resilient gels with very high flow points (Chapter 2, Table 2). The viscous nature of Fraction C could have, again, enabled easier diffusion of α -amylase, while the strong gel nature of the other fractions could have formed a specific intertwined tri-unit structure consisting of fibre network with embedded swollen and ruptured starch granules and leached starch polymers in aqueous environment. It is worth noting that when the starch is incorporated into the fibre gels and heated, the gel could have pushed the leached amylose onto the surface of the starch granules which would allow for easier enzymatic attack. As the preparation method specified in this work (Section 2.2.) consisted of firstly solubilising the fibre, followed by cooling, which allowed for gel formation, before adding the starch and

heating, it is possible that there was an increase in the effective concentration of amylose and α -amylase could explain the significantly higher hydrolysis rate along with the unchanged hydrolysis extent.

Even though these scenarios are possible, it is important to note that the experimental setup of starch hydrolysis did not take into account samples in the gelled state, rather, they were diluted. Moreover, they were homogenised which would have destroyed the gel matrix on a macroscopic level. Therefore, other mechanisms might provide better understanding of the increase of hydrolysis rate. One such occurrence is the fact that fibre addition can reduce crystallinity and induce disordered state of gelatinised starch (Gao et al., 2024; Qadir & Wani, 2022), which is more easily digested than ordered and crystalline structure. In addition, as mentioned in Section 3.3, the fibre could have also prevented retrogradation, and non-retrograded starch is more susceptible to enzymatic hydrolysis (Colonna et al., 1992; Patel et al., 2017; Slaughter et al., 2002). These mechanisms are relevant both for viscous and non-viscous fibre, and should be further explored by using x-ray diffraction and differential scanning calorimetry.

In addition, some mechanisms are applicable to only one type of fibre. Viscous and gel-producing fibres can enhance granule swelling, which would also mean easier enzymatic attack due to increased granule size and surface area. Due to their water absorbing properties, fibres can facilitate water diffusion into the granules, particularly at high temperatures when their viscosity decreases. Thickening properties of fibres also can slow down mobility of water molecules, which might form an “aqueous layer” surrounding starch granules, again facilitating hydration and swelling of starch granules. When subjected to mixing (as is the case in this work), viscous fibres can induce shear-thinning effect which also allows for easier hydration. On the other hand, non-viscous fibres (which are the PL fractions in this work) can lead to weaken macro- and micro-structure of the starch-fibre gels, making them weaker and less robust, thus the diffusion of amylase can be faster. Additionally, it is possible that the fibres might have interacted with the enzyme itself, however, that was not explored here and would be recommended for future areas of this work.

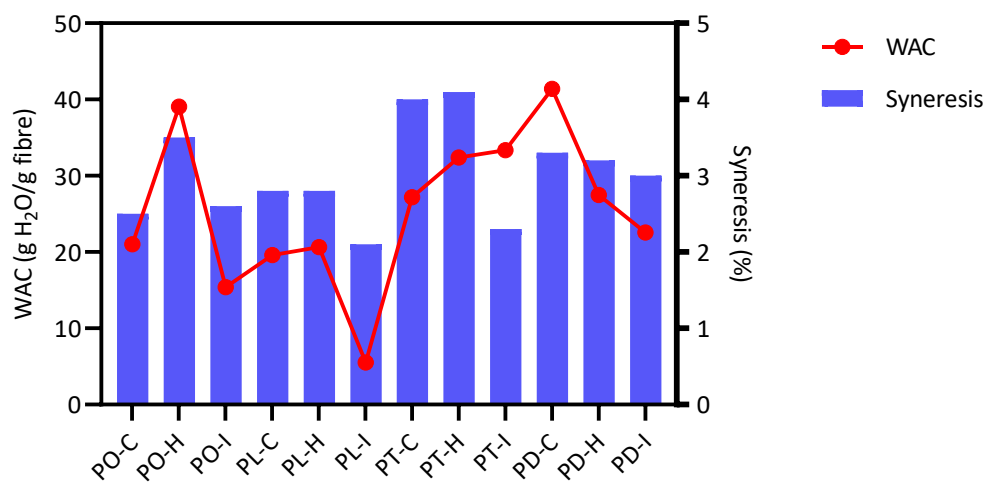
Overall, *Plantago* fibre fractions (excluding PO-H) unexpectedly showed adverse effects on the starch digestibility, especially the rate of hydrolysis. However, these findings open opportunities for further research, as addition of *Plantago* could still have beneficial effects, such as prebiotic activity and should be included in starch-rich food products. Different levels of fibre addition should be explored, as fibre quantity affects structure and digestibility of starch. Moreover, heat-moisture treatment with longer time and decreased amount of H₂O could yield different results and further insight into mechanisms. Gels studied in this work had very high H₂O content (94%), whereas such high hydration would be unlikely for most food application purposes. Method of preparation (fibre was dissolved first and, subsequently, starch was added and heated) can also be adjusted, which could also influence starch digestibility and retrogradation. To probe into possible mechanisms of starch hydrolysis and how does *Plantago* fibre affect starch structure, microscopy is recommended, specifically microscopy which would enable fluorescent labelling of fibre and enzymes, such as labelling with fluorescein thioisocyanate.

4. Conclusion

In this study, we explored three fibre fractions extracted from four diverse *Plantago* species (*P. ovata* (commercial psyllium), *P. turrifera*, *P. lanceolata* and *P. drummondii*) with varying chemical and rheological profiles (Chapter 2) as an addition to rice starch. The appearance of the gels which included fraction extracted with cold water (25 °C) were favourable, as they produced gels with lighter colour. All fibre fractions decreased freeze-thaw syneresis showing that they improved storage stability. Mechanical spectra (frequency sweeps) showed that all gels were classed as weak gels, and that the fibre addition mostly increased the viscous portion of the rheological behaviour, while amplitude sweeps showed increased viscoelastic moduli in all gels except those containing *P. lanceolata* fibre. Fibres from *P. ovata*, *P. turrifera*, and *P. drummondii* decreased yielding point, but increased flow point, suggesting that they increase gel resilience when the structural starch network is already compromised. Interestingly, starch hydrolysis by α -amylase showed undesirable

effects upon *Plantago* fibre addition, as all except hot water fraction from *P. ovata* (extracted at 65 °C) increased the rate of hydrolysis, which we hypothesis to be linked to the changes in gel structure and the delay of retrogradation. This study is the first to explore the effect that the incorporation of fibre fractions from multiple *Plantago* species into a food-like matrix has on product quality. Overall, two underutilised Australian species, *P. turrifera* and *P. drummondii* showed potential for this purpose, as their addition positively modified starch gel behaviour and their fractions were comparable to *P. ovata* regarding the appearance, demonstrating that they might be excellent alternative sources of hydrocolloids. The single fibre concentration used in this study is admittedly a limitation of the work and so future studies will investigate concentration effects of key fibres and the effect of different hydrothermal treatments. Studying microstructure by microscopy along with determining proportions of leached amylose may shed light on some of the hypotheses we have proposed. While this study introduced many hypotheses that are yet to be confirmed, the results highlight that *Plantago* fibre significantly affects structure and functional properties of rice starch gels in ways that may be further exploited in food industry.

Supplementary Figure 1. Syneresis of RS-fibre gels (blue columns, right y-axis) superimposed with water holding capacity (WAC) of each fibre fraction (red line, left y-axis).



Supplementary Table 1. Pearson's correlation matrix for rheological properties and starch hydrolysis parameters. Bolded value is significant at $p < 0.05$. Abbreviations: hydr.=hydrolysis, FT=freeze-thaw, CP=crossover point, YP=yielding point, FP=flow point, FTI=flow transition index. A' , b' , A'' , and b'' are power law fitting parameters for frequency sweep.

	FT syner esis	G'	G''	Tan δ_{LVER}	CP	YP	FP	FTI	A'	b'	A''	b''	A''/A'	b''/b'	Hydr. rate	Hydr. exten t
Hydr. rate	-0.34	-0.33	-0.39	-0.12	-0.29	-0.28	0.10	0.17	-0.29	-0.18	-0.41	0.14	-0.18	-0.32	1	0.29
Hydr. extent	-0.35	-0.36	-0.53	0.01	-0.51	-0.58	-0.46	-0.15	-0.29	-0.19	-0.50	0.29	-0.13	-0.40	0.29	1

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CHAPTER 5

Competitive gelation between starches and psyllium fibre (*Plantago ovata* mucilage): the effect of composite structure on rheology and freeze-thaw stability

Title:

Competitive gelation between starches and psyllium fibre (*Plantago ovata* mucilage): the effect of composite structure on rheology and freeze-thaw stability

Authors:

Lucija Strkalj^{1,2}, James M. Cowley², Rachel A. Burton², Gleb E. Yakubov^{1*}

¹Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom

²School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

*Corresponding author

Highlights:

- *P. ovata* mucilage has protective effect on viscoelastic profile of starch.
- Fibre-starch gel structure was highly dependent on amylose content.
- *P. ovata* mucilage and waxy corn starch showed antagonistic effect.

Abstract:

Composites of *Plantago ovata* mucilage (POM) with five starches of different composition (amylose %) and physical properties (particle size) show distinct behaviour (room temperature stability and freeze-thaw stability) which can be explained by their structure and rheological properties. POM, and five POM-starch composites have been studied at day 0 (freshly made), after 5 days at room temperature and after 5 days of freeze-thawing treatment. Amplitude sweeps have shown protective effect of POM application on mechanical properties of starch composite gels, especially after freeze-thawing.

The mechanism of the formation of a composite and an occlusive film rests on the competitive interaction between POM and starches for physical space and water. Specifically, POM and waxy corn starch show a distinct competition for space, which is not noted in starches with amylose. The balance between these effects dictates the degree of occlusive functionality and its impact on composites' microstructure.

The results demonstrate a prominent functionality of POM for delivery of high resistant starch formulations with superior techno-functional properties, including mechanical resistance, freeze-thaw stability and syneresis.

Keywords:

Plantago ovata mucilage, starch-fibre composite gels, starch-fibre interactions, rheology, syneresis

Abbreviations:

CS = corn starch, FT = freeze thawing, HAS = high amylose starch, NSP = non starch polysaccharides, NTT = non thermally treated, POM = *Plantago ovata* mucilage, RS = rice starch, RT = room temperature, SPS = soluble potato starch, TT = thermal treatment, WMS = waxy maize starch

1. Introduction

Starch hydrogels are widely used in food industry as there are diverse sources of starches with correspondingly diverse techno-functional properties that can be implemented as binding agents, thickeners, hydrocolloids, stabilizers, and fat replacers in various food products and packaging materials (Cheng et al., 2021). However, there are certain limitations to starch application in food products. Starch, when hydrothermally treated (i.e. gelatinised), becomes easily digestible and can cause rapid rise of blood glucose level which can lead to the insulin resistance and metabolic syndrome (Lal et al., 2021). During storage, a process called retrogradation occurs due to reorganisation of the amylose and amylopectin network (the two components of starch) and causes phase separation and syneresis - expulsion of water from the matrix of starch hydrogels which generally leads to the undesirable changes in starch-rich food products. Heterogeneity of starch hydrogels also contributes to the phase separation and syneresis (Koev et al., 2020). The differences in organisation of the starch matrix as caused by retrogradation are evident as changes in gel rheological properties, which are useful for assessing the influence of additives and processes on retrogradation of food products containing starch and to examine the fundamental mechanisms driving changes in the amylose and amylopectin network. Moreover, retrograded starches are technically more resistant to starch digestion and thus have a lower glycaemic responses which is desirable from a health perspective, but they also often have undesirable sensory properties so understanding and manipulating retrogradation is important for food and health applications (Baixauli et al., 2008).

Several strategies for maintaining the physicochemical and nutritional quality of starch hydrogels are available, but arguably the most common is the addition of non-starch polysaccharides (NSPs) like gums, fibres and hydrocolloids to create composite systems. For example, Lee *et al.*, (2002) compared nine different polysaccharide gums and demonstrated a wide impact on the freeze-thaw stability of sweet potato starch gels. The addition of guar gum (0.6%) showed the lowest syneresis (21.8%), followed by alginate (41.2%) and xanthan (46.8%), which significantly improved freeze-thaw stability of sweet potato starch whose syneresis was 71.3%. The proposed

mechanisms of how NSPs influence starch gel stability are varied. In one study, the addition of konjac glucomannan to corn starch improved freeze-thaw stability and increased viscoelastic moduli, attributed to entanglements between amylose and konjac glucomannan chains, which enabled formation of three-dimensional gel matrix and decreased mobility of starch chains, thus lowering the degree of syneresis (Ma et al., 2019). Rong *et al.* (2022) have studied the effect of xanthan, guar, and *Mesona chinensis* Benth gums in pea starch gels and reported increased pasting temperature, strain resistibility, elastic properties and hardness, and retarded disintegration in heating environment, which the authors attributed to the compact structure of pea starch-hydrocolloid matrix formed by hydrogen bonds. According to Chen *et al.* (2014), pullulan suppressed rice starch gelatinization and decreased viscoelastic moduli, but its effect was dependent on the concentration – at high concentration of pullulan (0.07% - 0.5%), amylose-pullulan interactions could hinder amylose-amylose interactions, but at a lower pullulan concentration (0.01% - 0.07%), it could not cover starch granules, which then swelled during gelatinization process and, consequently, increased the viscosity. One review concluded that the addition of NSP improves physicochemical properties of starch gels by forming NSP-amylose networks, and by absorbing the released water during freeze-thawing process, thus lowering the phase separation and syneresis (BeMiller, 2011). However, it is important to highlight that different NSPs might interact with various starches through multiple mechanisms, hence exploring a variety of NSPs with different molecular weights, charges, structures, and concentrations is key to elucidating their mechanism of action and in developing composite starch gels with targeted physico-chemical and health benefits.

Plantago ovata Forssk. (psyllium, isphagula, isabgol) is a myxospermous plant whose seeds have a layer of dehydrated fibre on the surface, which upon contact with water swells and forms a mucilage gel (Phan et al., 2020). *Plantago ovata* mucilage (POM) has an intricate layered structure, with more soluble pectic and heteroxylan (complex polymers of arabinose and xylose with a xylose backbone) mixtures in the outer layers that expand away from the seed, and pectin-depleted heteroxylan-rich layer(s) closer to seed, whose densely branched heteroxylans provide structural integrity to

mucilage (Phan et al., 2020; Yu et al., 2017). POM heteroxylans are bottle-brush polysaccharides that have short sidechains with a degree of backbone substitution higher than 90%. Current scientific consensus states the structure of POM heteroxylans as β -(1,4)-linked-D-xylopyranose backbone, heavily substituted at O-2 and/or O-3 positions with mono-substitution with α -L-arabinofuranose and β -D-xylopyranose, a di-substitution with α -L-arabinofuranose, and mono-substitution with oligosaccharides consisting of α -L-arabinofuranose and/or β -D-xylopyranose (Edwards et al., 2003; Fischer et al., 2004; Guo et al., 2008; Yu et al., 2017). Yu et al. (2017, 2018, 2019, 2021) have extensively studied the chemistry, microstructure, rheological properties, and gelling properties of POM and found very similar composition and linkage of inner POM layers, but markedly different rheological profiles, which are attributed to differences in sidechain distribution and hydrogen bond formation. This previous work has focussed on how POM fractions form different homotypic interactions (between *Plantago* polymers) but there are still outstanding questions regarding how POM's unique properties influence heterotypic interactions (i.e. interactions between POM and other macromolecules), especially with polymers relevant to the food and medical industry.

POM is a commercially valuable ingredient, with the husk and powder readily available to buy and consume as health-promoting supplements (McRorie & McKeown, 2017; Pal & Radavelli-Bagatini, 2012) and as a functional ingredient for gluten-free products (Ren, Linter, et al., 2020). Moreover, *Plantago* seeds are the only type of mucilaginous seeds whose husk can be separated which facilitates its commercial usefulness (Cowley & Burton, 2021). Noguerol et al. (2022) have characterized the gels from psyllium husk and powder, and suggested that they can be used in many applications as a clean label texturisers and fat replacements. Edible food films have been formed from POM combined with other biomaterials, such as whey protein isolate (Zhang et al., 2020), wheat acetylated starch (Askari et al., 2018), potato starch (Krystyjan et al., 2017), whey protein and modified lotus rhizome starch (Sukhija et al., 2019) and whey protein and yam starch (Sukhija et al., 2018). The results have shown that POM inclusion improved mechanical properties like tensile strength, decreased water vapor permeability, higher opacity, and improved thermal

properties. Belorio *et al.* (2020) compared psyllium to xanthan gum (an extremely common non-clean label thickener) in maize starch composites and concluded that they are comparable in hydration, pasting, rheological and textural properties meaning they might be a direct clean label alternative. Namely, psyllium can be added to yoghurts as fat replacer, and it can decrease syneresis and improve quality characteristics (Ladjevardi et al., 2015).

Even though POM is a promising addition for targeted development of starch hydrogels, surprisingly little is known about mechanisms of interactions between starch and psyllium fibre. Furthermore, POM represents a unique gel system due to two reasons. Firstly, 90% of POM is heteroxylan, which is unusual when compared to other myxospermous plants, such as Arabidopsis, flaxseed or chia, whose mucilage contain much higher amount of pectins (Cowley & Burton, 2021; Phan & Burton, 2018). And secondly, POM heteroxylan creates a physical gel due to hydrogen bonds between its densely branched structure, which is unlike other types of arabinoxylans, such as wheat, rye, or corn, whose ferulic acid residues enable formation of crosslinked chemical gel (Niño-Medina et al., 2010).

In this work, exploration of a promising fraction from *P. ovata* mucilage extracted at 65 °C is continued (Chapter 4, referred to as PO-H) due to its promising effect on rheology and starch hydrolysis. Monosaccharide composition of PO-H (Chapter 3) showed that it is primarily arabinoxylan (76.36% xylose and 17.70% arabinose) with very minor pectin component (1.7% pectin). The arabinose to xylose ratio was 0.23. The addition of PO-H showed the highest increase G' of rice starch (increase was 185.74%, Chapter 4, Table 2). This increase in the elastic portion of viscoelastic material is a compelling result for further study. In the previous chapter, 9 out of 12 fraction increased G' but the PO-H addition resulted in the highest increase, even though PO-H on its own (1% hydrogel) did not have the highest G' (chapter 3). Moreover, PO-H significantly reduced starch hydrolysis rate (rice starch = 0.038, rice starch-PO-H = 0.015 min⁻¹, Chapter 4, Fig. 5). More interestingly, it was the only fraction which decreased the starch hydrolysis rate. This results could not be explained by rheology alone, therefore additional experiments need to be carried out. To target the interactions between this distinctive bottle-brush polysaccharide

and starch, the described heteroxylan-enriched fraction was used for fabrication of composite gels with five different starches of varied chemical and physical properties. The influence of POM on gel storage stability, microstructure, and rheological profiles is investigated, and possible interaction mechanisms in the context of the dependency of starch type used are explored.

2. Materials and methods

2.1. Materials

P. ovata seeds were purchased at a local shop in Nottingham, UK. Five different starches were used in experiments: soluble potato starch (S-2004, Sigma Aldrich, UK), native rice starch (Remy DR, Beneo, Germany), waxy maize starch (AmiocaTM TF, Ingredion UK Ltd, UK), corn starch (S-4126, Sigma Aldrich, UK), and high amylose maize starch (HylonTM VII, Ingredion UK Ltd, UK). In the further text, these abbreviations will be used: soluble potato starch – SPS, rice starch – RS, corn starch – CS, waxy maize starch – WMS, and high amylose maize starch – HAS. These starches are chosen as they represent a variety of different starch ghost structure (explained in detail in section 3.1.) which would allow for more thorough understanding of how *Plantago ovata* mucilage fraction will interact with different starches.

Sodium azide (S2002) was purchased from Sigma Aldrich (Australia).

Amylose content of starches was determined by 96-well plate iodine binding assay (Kaufman et al., 2015).

Particle size of starches was determined using a LS 13 320 laser diffraction Particle Size Analyser (Beckman Coulter, Meritics, Dunstable, UK) with a dispersion cell (universal liquid module) filled with water to disperse the samples. The diffraction data was analysed with the equipment software using the Fraunhofer model. Moisture content was determined with MB90 Moisture Analyzer (Ohaus Corporation Europe GmbH, Switzerland). Moisture content of starches was as follows: SPS – 7.28%, RS – 10.83%, CS – 11.12%, WMS, 11.52% and HAS – 10.67%.

For the fluorescent microscopy, Calcoflour-white (18909, Sigma Aldrich) (0.25 g/L) and fluorescein isothiocyanate (F7250, Sigma Aldrich) (0.1 g/L) were used to stain the gels.

2.2. Methods

2.2.1 *Plantago ovata* mucilage (POM) preparation

Mucilage extraction was done according to Yu *et al.* (2017). Briefly, 20 g of *P. ovata* seeds were dispersed in 800 mL water and stirred (200 rpm with magnetic stirrer) for 4 h at 25 °C. The mixture was then centrifuged at 10 000 g for 45 min at 25°C (Beckman J2-21 centrifuge, rotor JA-10, IN, US) and the supernatant was discarded. The pellet (which consisted of seeds and gel) was re-dispersed in 800 mL water and stirred for 4 h at 65 °C. The mixture was centrifuged at 5000 g for 45 min at 65 °C. The pellet was discarded, and supernatant (which consisted of water and dissolved fibre) was dialysed against water for 24 h using 12-14 kDa MWCO dialysis tubes. After the dialysis, the gel was freeze dried for 1 week (Freeze Dryer Modulyo, Edwards, York, UK) and freeze-dried material was used for further experiments. This fraction is hereafter referred to as POM (*Plantago ovata* mucilage).

2.2.2. Gel preparation

Gels were prepared as follows: sodium azide was dissolved in H₂O at a concentration of 0.05 w/w, after which the POM was dispersed in H₂O (1% w/v) and homogenised using an Ultra-Turrax (T25, Ika®-Werke, Germany) at 1200 rpm for 1 min. The POM dispersion was then stirred (200 rpm) at 65 °C for 30 min. When preparing POM-starch gels, 5% w/v (dry weight) starch was added to the POM dispersion, homogenised for 2 min, and heated to 90 °C for 10 min. Starch only gels were prepared by dissolving 5% w/v starch in H₂O and heated to 90 °C for 10 min.

Overall, 9 different gel types were prepared: POM gel, 5 different composite POM-starch gels, and 3 different starch gels. The 3 starch gels were: corn starch, rice starch and waxy maize starch. Soluble potato starch does not gel at the concentration used in the experiments (5% w/v), while high amylose starch requires harsher conditions (temperatures >90 °C, for example) to gelatinise.

To further test POM-HAS gel structure (explained in 3.4.) rheological experiments (protocol in 2.2.3.) of POM-HAS gel with different concentrations of HAS (1, 3, 5, 7 % w/v) following the same protocol as previously described were conducted. Furthermore, the POM-HAS (HAS 5% w/v) structure is tested by doing the following: the protocol for gel preparation was conducted as previously described, POM-HAS gel was then centrifuged 30 min at 90 °C to keep the temperature conditions of gel preparation stage, the upper layer (POM) was used for rheological experiments (ch. 2.2.3.), while the bottom layer (HAS) was discarded.

2.2.3. Room temperature and freeze-thaw syneresis

Syneresis of gels was measured using two methods: at room temperature and at freeze-thawing conditions. Approximately 10 mL of gels were transferred in 50 mL centrifuge tubes, left at room temperature, and 5 days after supernatant was carefully decanted. Mass of gels and tubes was measured before and after supernatant was decanted. Freeze-thawing syneresis method was performed according to Ma et al. (2019) without the centrifugation. Briefly, gels were kept at - 20 °C for 24 h, and then thawed at 40 °C for 2 h. This cycle was repeated 5 times, after which supernatant was decanted and remaining gel was weighed.

2.2.4. Fluorescent microscopy

Gels were stained with mixture of Calcoflour white (0.25 g L^{-1}) and fluorescein isothiocyanate (0.1 g L^{-1}). Micrographs were taken by fluorescence microscope (EVOS FL, Waltham, US) equipped with DAPI (357/44 – 447/60 nm) and GFP (470/22 – 525/50 nm) light cubes.

2.2.5. Amplitude oscillatory rheological experiments

Rheological experiments were performed on MCR 301 rheometer (Anton Paar, Austria) with sandblasted parallel plate geometry (PP40-SN11649, Anton Paar). The measuring gap was 1 mm. Extra sample was trimmed by a spatula and the edge of samples was covered by low viscosity mineral oil (Sigma, USA) to prevent drying of samples. The temperature was controlled by a Peltier system with the assistance of a water bath (R1, Grant, Shepreth). Amplitude sweep experiments were determined at 0.01 to 1000 % strain rate and at 10 rad/s frequency and 25 °C temperature.

Amplitude sweep was determined on freshly prepared gels, gels after 5 days of room temperature syneresis, and gels after 5 days of freeze-thawing cycles. All gels were equilibrated at room temperature prior to measurement.

2.2.6. Statistical analysis

All measurements were conducted in triplicates, and presented as mean $\pm$ standard deviation. OriginPro 2022b software was used to perform one way ANOVA with post-hoc Tukey test ($p < 0.05$).

3. Results

3.1 Starch characteristics

To examine the influence of POM on different range of starches and to elucidate its effects based on starch properties, 5 types of starch are chosen based on the distinct differences their amylose to amylopectin ratio, particle size, and gelatinised structure. Fig. 1 shows amylose content of each starch (Fig. 1A) and particle size (Fig. 1B). Literature has shown that amylose and amylopectin ratio affects interaction with NSPs (BeMiller, 2011). As expected, HAS had the highest amylose content (68.30%), and WCS the lowest (1.63%), while conventional corn starch showed 29.87% amylose, agreeing with previously published results (Beech et al., 2022). Soluble potato starch had 26.00% amylose, and rice starch slightly lower (22.63%).

Particle size is another parameter of importance when assessing the starch properties. All starches had unimodal distribution (Fig. 1B). D(50) is median particle size. RS and HAS had the two lowest d(50) parameters which were 8.48 and 9.36 μm , respectively. Larger particle size was measured in CS (d(50) = 15.51 μm) and WMS (d(50) = 14.59 μm). SPS had the largest particle size (46.09 μm).

After gelatinization, the starch granules form starch ghosts, which is an insoluble structure of gelatinised starch (Li & Wei, 2020). This is an important occurrence as it is directly related to its functionality and starch-NSP interactions. Swelling power and water absorption capacity are parameters which help us understand the behaviour of a starch when subjected to hydrothermal treatment. Swelling power of corn starch

was reported to be 8.70-15.00 g/g, and water absorption capacity was 1.98-2.32 g/ml (Moses & Olanrewaju, 2018). Waxy starches have higher swelling power due to the higher content of amylopectin, which has highly branched structure and can absorb more water. Waxy corn starch showed swelling power of 21.7 g/g, while its four conventional corn starch counterparts all had swelling power lower than 19.4 g/g (Sandhu et al., 2008). Rice starch showed swelling power of 8.3 g/g (Kong et al., 2015). Soluble potato starch is highly soluble, and does not swell when heated. High amylose corn starch does not fully gelatinise under described conditions, and retains granular structure. Another important aspect to consider is the structure of POM-starch gels after they have been heated (90 °C, 10min), therefore different appearances of starch ghosts are present. For example, starch ghost of WMS would mostly comprise of short-chained amylopectin network and lower amount of long-chained amylopectin (Li & Wei, 2020). CS and RS are considered conventional types of starch regarding their amylose content (20-30%), but their starch ghosts would be different due to particle size difference. Owing to high amylose content, HAS usually needs to be hydrothermally treated at higher temperature than its conventional counterpart (Li et al., 2019) in order to completely gelatinise, so it is reasonable to assume that following the described protocol, HAS gelatinised structure would consist of starch granules and some amylose chains. Lastly, SPS is a type of starch which is soluble when gelatinised, thus it does not provide gelatinised network or starch ghosts, and NSP-SPS structural properties would depend on properties of NSP. Therefore, these five starches were chosen to form starch gels and starch-fibre gels as they show a range of possible structures and would provide more insights into interactions between the fibre and the starches.

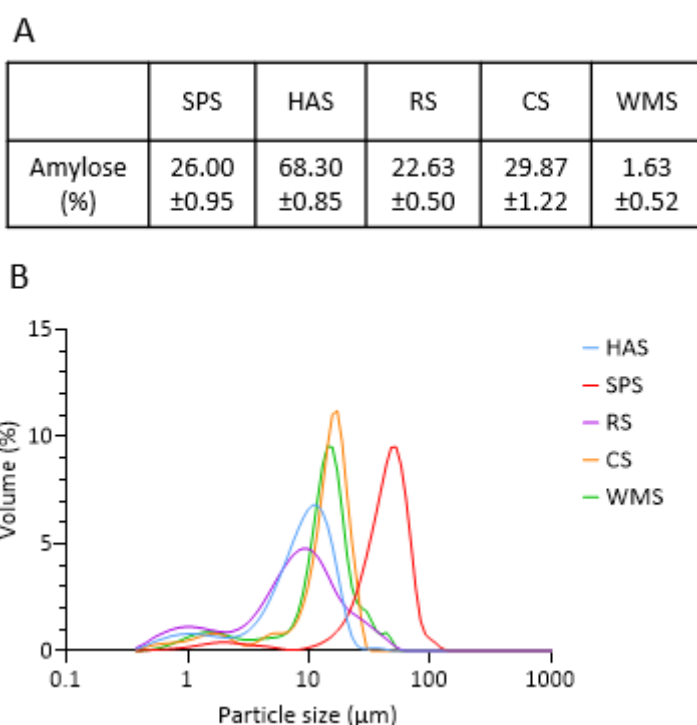


Figure 1. Physico-chemical starch properties. Figure 1A: amylose content (%) of starches presented as mean and standard deviation of three replicates. Figure 1B: particle size analysis of five starches.

3.2. Syneresis

Syneresis is a two-step process happening during starch retrogradation: the short term retrogradation is driven by amylose helices creating a compact network and forming hydrogen bonds, while the long term retrogradation is based on amylopectin chains forming a continuous network (Dobosz et al., 2020). The short-term retrogradation happens upon cooling of starch gel and is continuing for a few hours during the cooling process, while long-term retrogradation happens during a span of a few weeks (Dobosz et al., 2020; Funami et al., 2005). Both of these networks cause water expulsion during retrogradation, thus leading to syneresis. Fig. 2 shows the 9 different prepared gels, including 1% POM, after 5 days of storage at room temperature (5 days RT) and after 5 days of freeze-thaw cycles (5 days FT). The following starch-POM gels are compared: RS, POM-RS, CS, POM-CS, WMS, and POM-WMS. Since HAS and SPS do not exhibit gelation at previously described conditions, the syneresis results of their starch-POM gels are presented without their starch-only

“counterparts”. Syneresis measured using two different methods offers insight into the transformation of starch matrix, as well as impact of POM on starch network during these conditions.

Our results show relatively low syneresis when compared to some published literature, both for starches and NSP-starch gels. A probable reason for this is difference in syneresis protocols, as they are not standardized. The centrifugation step was omitted since it is proven that the centrifugation force affects the sample and level of syneresis (Abd Karim et al., 2000). Thus, it is more difficult to compare and interpret results from different studies. Moreover, since the gels were subjected to syneresis measurements (both RT and FT) and later to oscillatory rheology to assess the microstructure, the goal was to avoid potential disturbance of the gel microstructure.

As expected, all samples showed higher syneresis when subjected to FT, apart from the POM-WMS gel. During freezing, water forms ice crystals, which causes phase separation. Later, during de-frosting, starch gels have starch-rich and starch-depleted regions, depending on the location of ice crystals, and water from starch-depleted regions gets expelled easily. The continuation of multiple FT cycles accelerates phase separation and syneresis, as amylopectin retrogrades in starch-rich regions (Abd Karim et al., 2000). POM universally improved FT stability, as it decreased the degree of syneresis in RS by 81 %, in CS by 91 %, and in WMS by 58 %. Here, it can be assumed that FT cycles led to both short-term and long-term retrogradation, since repeated exposure to low temperature (-20 °C) followed by thawing (at 40 °C) causes phase transitions of water and additional starch retrogradation (Sae-kang & Supphantharika, 2006). Moreover, POM lowered syneresis in CS after RT storage, while RS and POM-RS had the same syneresis values after RT treatment. Psyllium fibre lowered syneresis in jellies and yoghurts (Figuerola & Genovese, 2019; Ladjevardi et al., 2015), but to best of our knowledge, no study has investigated the interaction mechanisms during syneresis of starch-psyllium gels. Different NSPs have been added to starch gels to examine their effect on the storage properties: the higher concentration of xanthan gum (0.2% compared to 0.05%) lowered syneresis of the potato starch with varying amylose content (Dobosz et al., 2020). Ma *et al.* (2019) have shown that high Mw

(molecular weight) konjac glucomannan has decreased FT syneresis of corn starch more than low Mw konjac glucomannan. There are a few proposed explanations on how the interactions between starch and NSPs can lower syneresis and preserve functionality. Ferrero *et al.* (1994) suggested that NSPs mostly hinder the process of amylose retrogradation, meaning that less hydrogen bonds between amylose chains are formed, and the structure is less crystalline and more disordered. This explanation has been expanded when observing effect of xanthan gum on tapioca starch pastes (Sae-kang & Supphantharika, 2006). The phase separation between xanthan gum and amylose led to less leached amylose during freeze-thawing cycles and, consequently, lower syneresis. Another explanation is that the water expelled from starch is re-absorbed by NSPs or is re-absorbed in porous structure of NSP developed gels (Dobosz *et al.*, 2020; Ma *et al.*, 2019).

Taking this into consideration, we propose an explanation about POM's effect on WMS, which is comprised of amylopectin. As mentioned, amylopectin is predominantly responsible for long-term retrogradation, where amylopectin chains (usually within the same molecule) form a continuous network, which causes syneresis. In 5 days of RT storage, the long-term retrogradation is most likely not yet completed (BeMiller, 2011), so the degree of syneresis of WMS will be low. When POM is added, this disrupts the amylopectin ghost structure and causes water to be expelled, due to the competition for space and thermodynamic incompatibility of the two polysaccharides. Thus, after 5 days RT, WMS gel remained stable and had lower syneresis than WMS-POM gel. However, 5 days of FT caused repeated phase transitions of water and long-term retrogradation, which affected amylopectin network and increased syneresis. As the water was being expelled, POM absorbed it and, consequently, POM-WMS had lower syneresis than WMS gel after FT treatment.

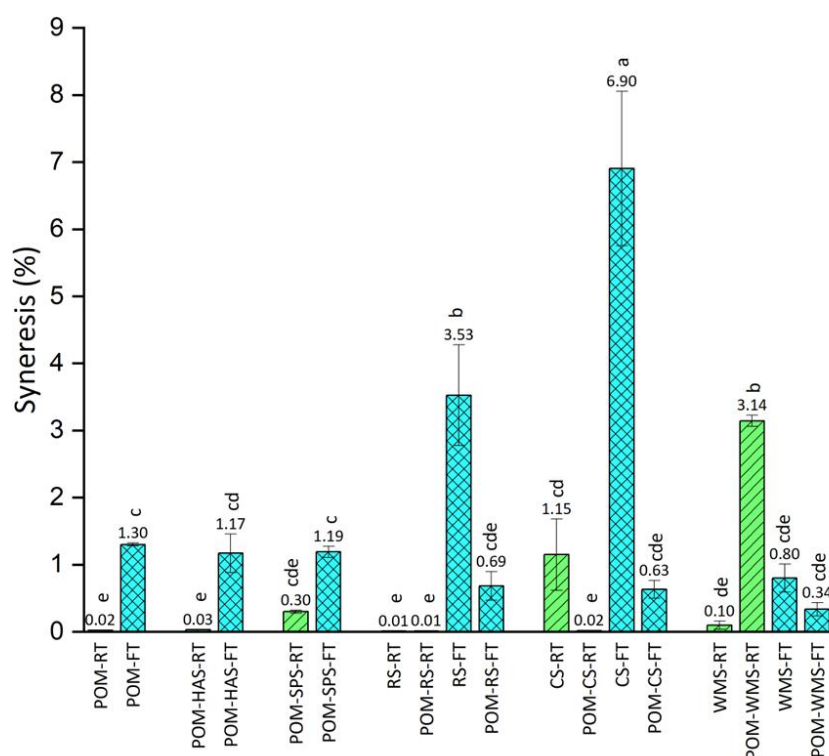


Figure 2. Room temperature syneresis and freeze-thaw stability (measured after 5 days) of POM, POM-SPS, and POM-HAS are shown, as well as RS, CS, and WMS with and without addition of POM. Green colour and diagonal pattern represented room temperature syneresis, while blue colour and crossed pattern represents freeze thaw syneresis. Values are mean of three replicates. Different letters mean statistically significant differences at $p < 0.05$.

3.2. Fluorescent microscopy

Fluorescent micrographs of POM-starch gels at 4 different conditions (non-thermally treated, thermally treated at day 0, after 5 days at RT, and after 5 days FT) are shown in Fig. 3. Starch is shown in green colour and it is stained with fluorescein isothiocyanate, while POM is shown in magenta colour and is stained with calcofluor white. Micrographs show microstructures unique to each starch type and its changes as a result of thermal treatment and storage. All non-thermally treated (NTT) gels clearly show dispersed starch granules in POM matrix, while thermal treatment (TT) caused changes in almost all gels. RT storage had a limited effect on visible structure, while FT storage caused consolidation of starches and POM.

POM-HAS gel has visible starch granules after TT, suggesting that HAS has not been fully gelatinised. It has been shown that the high amylose starches keep semicrystalline structure at conventional cooking processes and start to gelatinise at higher temperature than their conventional or waxy starch (Li et al., 2019). The same can be seen in our work as well – high amylose corn starch (HAS) retained granular structures after TT (Fig. 3B) whereas normal corn starch (CS) lost its crystallinity and shape (Fig. 3N). CS after TT displayed larger and more irregular size and shape, with visible starch granule ghosts. Moreover, POM-HAS retained its microstructure after both storage conditions (5 days RT and 5 days FT, Fig. 3D and 3E, respectively), which points to starch granules being present as particles in POM matrix. POM-SPS showed a loss of starch granules after TT. Since SPS is a highly soluble starch, it did not disturb POM matrix, as can be seen in the micrographs (Fig. 3E-H). POM-RS showed a loss of crystallinity after TT, whereas both TT and 5 days RT micrographs showed entrapment of small RS granules in POM matrix (Fig. 3J and 3K). POM-CS also showed loss of crystallinity, which is easily visible due to the larger granule size after TT (Fig. 3N). The aggregation of CS particles can be noticed after 5 days of RT (Fig. 3O). Both POM-CS and POM-RS were incorporated with the POM matrix after 5 days RT, and, after 5 days of FT, both gels showed consolidation, as starch no longer has granular shape and green colour is almost grey (Fig. 3J and 3P), as a result of superimposition of green (starch) and magenta (POM) (Chen et al., 2014). On the other hand, POM-WMS showed different microstructure after TT (Fig. 3S) and after 5 days RT (Fig. 3T). There are no visible starch granules or starch ghosts, rather it seems that POM matrix is almost disrupted and fragmented (Fig. 3T). Since waxy maize starches are made of >99% amylopectin, which forms its own network, we propose that POM and WMS ‘compete’ for space in the matrix, where both polysaccharides form and extend individual spatially separated networks without synergistic interactions between the two due to thermodynamic incompatibility (BeMiller, 2011). However, after 5 days of FT, POM-WMS gel structure is similar to POM-RS and POM-CS, indicating consolidation of POM and WMS. Along with the microscopy showing bicontinuous phase structure (Fig. 3U), the theory is also supported by the syneresis results whereafter 5 days of RT, POM-WMS had higher syneresis than WMS alone implying phase separation. After 5 days of FT, POM-WMS gel had statistically insignificant, but

still lower syneresis than WMS alone, indicating some consolidation with POM. The differences in gel microstructure can be noticed when applying tamarind seed gum in corn starches with different amylose content (Xie et al., 2020) as well, and can be comparable to our results presented here – WMS had continuous network which was then changed to compacted when tamarind seed gum was added, as opposed to granular structure of HAS and CS.

Microscopy showed interesting findings and once again pointed out the differences in POM-starch gels governed by starch type, therefore, the following rheology results complete and expand the proposed explanations of structure properties and interactions between POM and starches in composite gels.

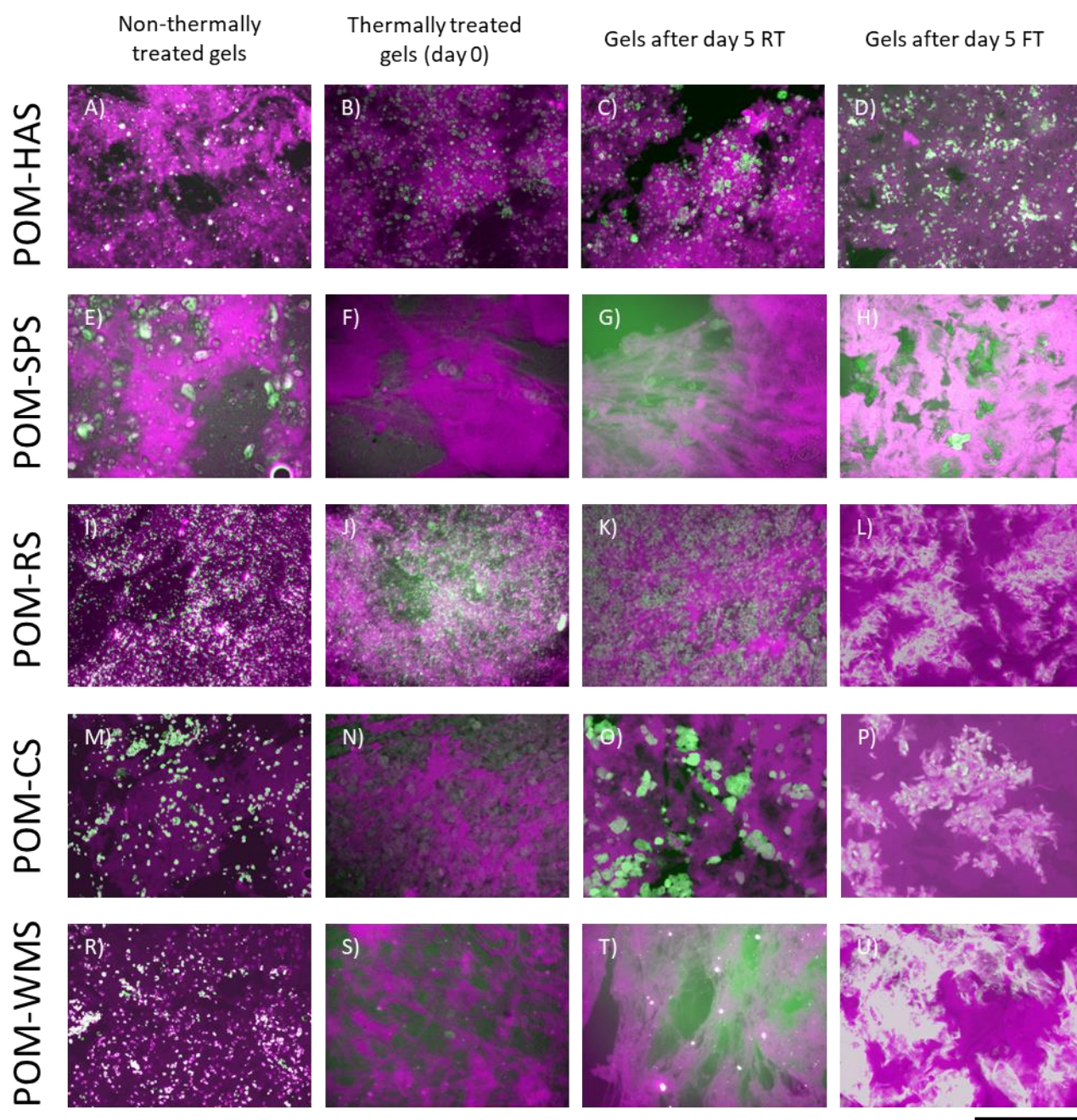


Figure 3. Micrographs of 5 different gels showing starch (green) and POM (magenta) at 4 different conditions: non thermally treated gels, thermally treated gels (freshly prepared at day 0), gels after 5 days of RT syneresis (day 5 RT) and gels after 5 days of FT syneresis (day 5 FT). Scale bar represents 400 μ m.

3.4. Rheology

In Fig. 4, the oscillatory amplitude sweep of 9 different gels is presented, including 1% POM, at the three previously described conditions: Day 0 = measured 2 hr after preparation, Day 5 RT = after 5 days of room temperature storage, and Day 5 FT = after 5 days of freeze-thawing cycles. The following starch and POM-starch gels are compared; RS, POM-RS, CS, POM-CS, WMS, and POM-WMS. Since HAS and SPS do not exhibit gelation at previously described conditions (Section 2.2.), the rheological properties of HAS-POM and SPS-POM are presented without their “counterparts”. Moreover, HAS-POM rheological behaviour is examined more thoroughly by utilising a range of concentrations and preparations, in an attempt to unpick some of the possible mechanisms. The gels have strikingly different rheological profiles, even though they have same solids concentrations (6% in POM-starch gels, and 5% in starch gels), and have different behaviour after synereses. This is an indication of the unique interactions between starches and POM, depending on starch type.

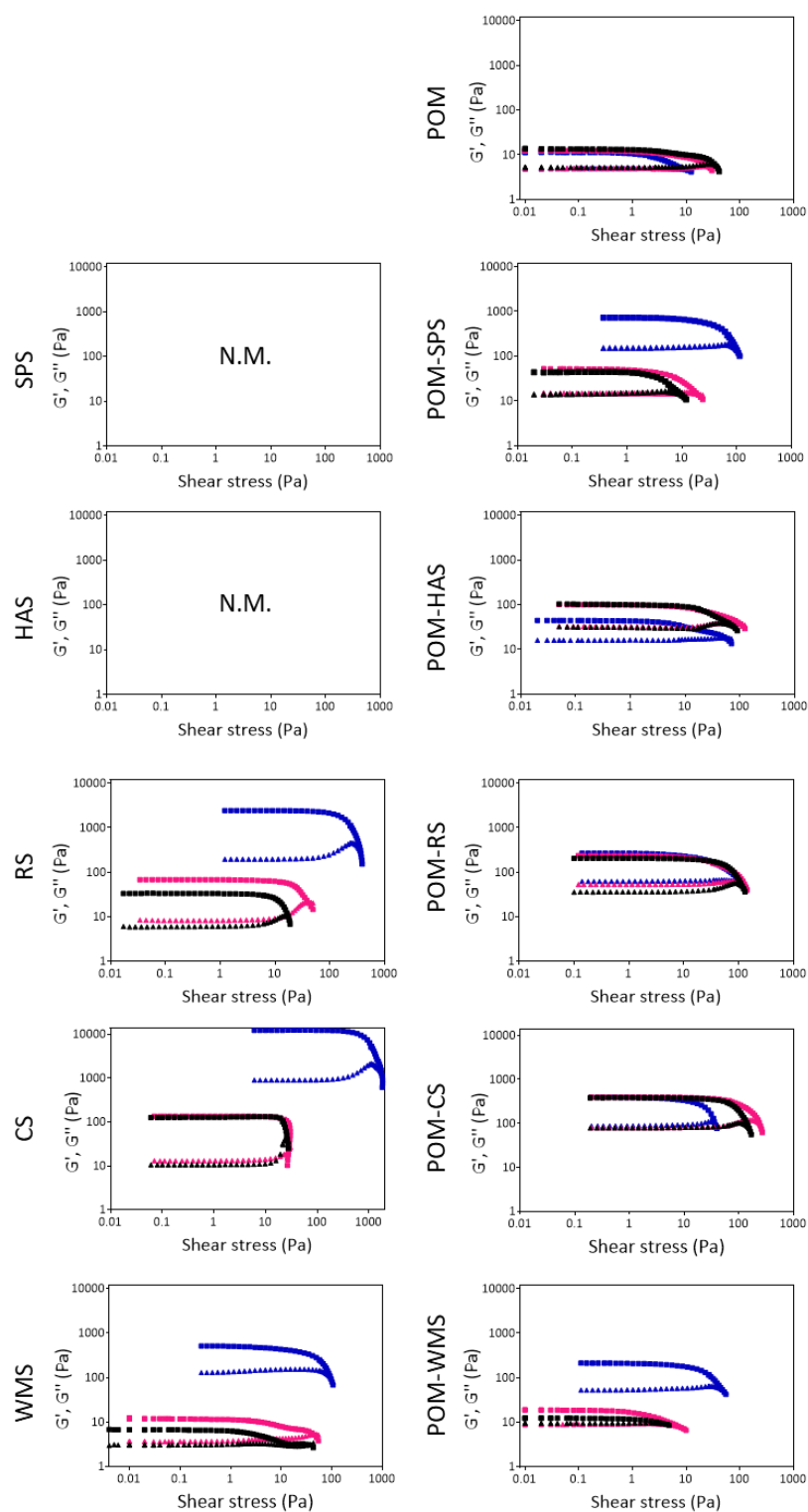


Figure 4. Amplitude sweep of 9 gels shows storage modulus (G') and loss modulus (G'') as dependent on shear stress (Pa). G' is represented by squares, and G'' as triangles. All gels are shown at 3 conditions: day 0 (black colour), after 5 days of room temperature storage (pink colour), and after 5 days of freeze-thawing cycles (blue colour).

Amplitude sweep shows $G' > G''$ in linear viscoelastic range of all samples, confirming they are viscoelastic gels (Fig. 4). The addition of POM increased viscoelastic moduli when it was added to all the starch gels (starch < POM-starch), apart from when added to WMS (WMS > POM-WMS). Usually, non-starch polysaccharides (NSP) cause higher G' and G'' in starch gels due to two reasons: 1) increased amount of solids in the sample, and 2) interactions between starch chains and NSP (BeMiller, 2011). As an example, NSPs added to 8% pea starch gel increased G' (xanthan gum > guar gum > *Mesona chinensis* Benth gum) (Rong et al., 2022). The authors attributed the increase in G' to formation of a pea starch-NSP three-dimensional network, strengthening effect of NSPs, and promotion of chain-chain interactions due to increased solids concentration in gel and lower water activity. Usually, starch-starch chain interactions are responsible for structure and properties of starch gel, but with the addition of NSPs, starch-NSP interactions are considered to produce a denser network. NSPs can cover the surface of starch granules, which prevents water loss and amylose leaching. Moreover, NSP preserve viscoelastic profiles during storage by preventing network degradation. This means that, during storage, composite gels show lower increase in G' than starch gels (Dobosz et al., 2020; Kim & Yoo, 2006; Satrapai & Supphantharika, 2007), which is commercially desirable, and can be confirmed in gels studied in this work. When RS, CS, and WMS are compared to POM-RS, POM-CS, and POM-WMS, it is clear that composite gels had much lower increase in G' after storage, especially in freeze-thawed gels (Fig. 4 and Table 1).

G' of POM was 13.45 Pa, which did not change even after FT treatment, meaning the elastic portion of rheological behaviour has been preserved. Its crossover point (COP) was impacted by FT treatment – it decreased from 20.49 Pa (day 0) to 1.99 Pa (day 5 FT), thus the gel has lost its strength. Ren *et al.* (2020) have reported G' approximately 100 Pa in 1.64 % POM gel (fraction extracted at 60°C). Zhou *et al.* (2020) have shown rheological values of 1% psyllium seed husk polysaccharide extracted at 85°C, with G' being approximately 20 Pa. These variations can be attributed to different types of extractions (which lead to distinct gel compositions) and different concentrations of gels. Moreover, it was recently shown that weather conditions influence qualities of

psyllium husk like water retention and monosaccharide composition of POM (Cowley et al., 2022), thus it can be assumed that *P. ovata* grown in different conditions could produce mucilage with different rheological properties as well.

G' of POM-SPS gel slightly increased from day 0 to day 5 RT, and had a significant increase after 5 days FT (43.99 Pa, 51.78 Pa, 725.91 Pa, respectively). This indicates that gel became harder, which is a common occurrence after a gel has been exposed to freezing and thawing.

When comparing viscoelastic profile of RS and POM-RS, it is clear that the addition of POM had a protective effect: even after FT measurement, G' and flow transition index of POM-RS had no statistically significant differences. This agrees with the trend seen in Section 3.2., where there was low amount of syneresis (POM-RS at FT = 0.69%) . On the contrary, G' of RS gels after 5 Days of FT syneresis was 73x higher than G' of RS gels on Day 0. It is important to highlight similarities and differences between POM-RS and RS-PO-H from our previous study (Chapter 4). The gel studied here had a G' of 204.52 Pa, while RS-PO-H had a somewhat lower elastic modulus ($G' = 90.47$ Pa). One key reason for this could be differences in fibre preparation: in this study, freeze-drying was used to obtain the fibre, while in Chapter 4, fibre was oven-dried at 95 °C, which could have had a slightly detrimental effect on its structure, and therefore, ability to form a strong gel when combined with rice starch. However, both gels had similar $\tan \delta_{\text{LVER}}$: RS-PO-C = 0.23, POM-RS = 0.18, indicating weak gel nature for both gels. Moreover, in both cases, $\tan \delta_{\text{LVER}}$ of RS-fibre gels were not significantly different from $\tan \delta_{\text{LVER}}$ of RS gel, indicating that their viscoelastic nature was not disturbed by fibre addition.

A positive effect of POM can be seen when applied to a CS gel. POM-CS gel showed no changes in G' during all 3 storage conditions, while crossover point was affected after FT storage (COP after FT storage was 4x lower than COP measured on day 0). This indicates that gel became more brittle. CS gel had the highest increase in G' after 5 days of FT of all samples – its G' after 5 days FT was 1000x higher than G' on Day 0. Rheology results support syneresis results: increase in G' and degree of syneresis are considered to be a measure of retrogradation (Krystyjan et al., 2022). In our case, CS after FT storage had highest G' and highest degree of syneresis, followed by RS, and

viscoelastic profiles corresponds to FT measurements. These findings show promising effects of POM addition into starch composite systems – preserving rheological profiles of starchy systems is necessary for overall sensory acceptance, and physical stability of food products during storage.

Protective effect is reported in other NSP-starch systems as well, during short-term and long-term storage. Galactomannan-rice starch gel showed lower increase in G' and G'' after 10 h storage at 4 °C than rice starch gel (Kim & Yoo, 2006). Increase of G' after aging was caused by thermodynamic incompatibility between starch and galactomannan, which caused phase separation and, consequently, increased the concentration of rice starch in the continuous phase. Next, β -glucan and rice starch composite gel were stored at 4°C for 60 days and displayed lower changes in G' and G'' , when compared to rice starch gel alone (Satrapai & Supphantharika, 2007). The authors attributed this difference to β -glucan ability to retard the recrystallization of amylopectin and mobility of starch chains, resulting in softer gel. It has been shown that a 0.2% addition of xanthan gum in three different types of potato starch gel decreased hardness during 90-day storage at 6°C (Dobosz et al., 2020). This agrees with our results – POM addition resulted in higher G' in all starch gels, except WMS (which predominantly amylopectin).

Dissimilar to other samples, POM-WMS gel showed specific rheological behaviour (much like syneresis results). Its G' was 12.28 Pa, which was slightly lower (though not statistically significant) than G' measured for POM alone (13.45 Pa). This is unusual, considering that POM-WMS gel had 6 % solids concentration, and POM gel was measured at 1 % concentration. $\tan \delta$ of POM-WMS was also higher than WMS gel (0.78, and 0.47 on day 0, respectively), which means that viscous behaviour was more characteristic for POM-WMS than WMS. This suggests that POM disrupts WMS network, facilitating amylopectin chain rearrangements when subjected to strain. Length of linear viscoelastic range (LVER) is another parameter related to structure stability. Long LVER suggests that measured sample is stable and well-dispersed (Mariotti et al., 2009). WMS had the longest LVER, followed by POM, whereas POM-WMS had shorter LVER. Moreover, WMS gel had the highest normalised yielding point and flow transition index of all samples, indicating that WMS alone forms

stronger gel than POM-WMS. These findings agree with our theory that WMS and POM mutually cause disruption of each other's networks, thus causing lower elastic moduli and weaker gels. After 5 days of RT storage, G' of POM-WMS was stable, even though syneresis was 3.14 % (highest of all samples measured at RT) (Fig. 5). This is another indication that POM and WMS polysaccharide chain have incompatible interactions, meaning that they behave antagonistically. However, protective effect of POM is reported after 5 days of FT: G' of POM-WMS was 211.88 Pa, and G' of WMS gel was 508.05 Pa.

Overall, the rheology results agree with previously proposed mechanisms of each composite gel, with the support of syneresis results and microscopy images. At Day 0, G' of POM-WMS gel was lowest of all composite gels, which agrees with published literature. When rice starch with varying amylose contents (from 0% to 28%) were studied with addition of different NSPs, it was found that G' increased as a function of increased amylose content (Kim et al., 2013). In our case, POM-HAS did not have highest G' , even though HAS is the starch with highest amylose content, which can be explained by different type of gelling system that POM-HAS displayed.

POM-HAS gel had G' of 102.11 Pa and G'' of 31.39 Pa. Storage at room temperature did not change G' and G'' , while FT syneresis decreased both values ($G' = 44.01$ Pa, $G'' = 16.03$ Pa). Other gels showed an increase in G' and G'' following both RT and FT storage, which agrees with published literature (BeMiller, 2011). However, decreases in viscoelastic moduli of HAS-POM after storage suggests that POM and HAS have most likely create a two-phase jamming structure, and not a network gel. This is noticeable in microscopy images as well – HAS granules are clearly visible at all conditions (Fig. 3). Therefore, another set of POM-HAS gels with 4 different concentrations of HAS were fabricated (1, 3, 5, 7 %). G' of POM-HAS gels are shown in Supplementary Fig. 1. It is shown that increasing concentration of HAS results in increased G' , but only up to HAS concentration of 5%. G' values of POM-HAS gels with increasing HAS concentrations (1, 3, 5, 7 %) are as follows: 10.7, 25.4, 97.4, and 45.3 Pa. Initial increase can be attributed to increased solids concentration, but at 7% HAS network was weakened, which led to lower G' . This occurrence can be seen in

emulsion gel models when inactive fillers weaken gel network as they act as small holes (Farjami & Madadlou, 2019).

Table 1. Values of G' , G'' , tan delta, normalised yielding point (nYP – yielding point divided by G' of the same sample), normalised crossover point (nCOP – crossover point divided by G' of the same sample), and flow transition index (FTI – flow point divided by yield point, which indicates tendency for brittle fracturing of a gel) are presented. All values shown are mean of three replicates and standard deviation (unless the standard deviation was <0.01). Different letters show significant difference between samples ($p < 0.05$). Small letters indicate significant difference between the same sample under the 3 conditions (for example, POM measured at day 0, day 5RT, and day 5FT). Capital letters indicate significant difference between all samples at the same condition (for example, all gels at day 5RT).

		G'	G''	tanδ	nYP	nCOP	FTI
POM	Day 0	13.63±0.85 aD	5.27±0.18 aDE	0.39±0.02 bc	0.09±0.01 bD	2.56±0.24 aB	28.02±0.21 aB
	Day 5 RT	12.31±0.30 abG	4.99±0.05 abF	0.40 bB	0.09 bc	2.18 aB	24.79±0.21 aA
	Day 5 FT	11.26±0.22 bC	4.96±0.02 bE	0.44 aA	0.12 aB	0.82 bB	6.63±0.15 bB
SPS	/	/	/	/	/	/	/
POM-SPS	Day 0	102.20±8.93 aC	31.41±2.49 aB	0.31 cD	0.14 bcd	0.75±0.13 cbC	5.42±0.94 bC
	Day 5 RT	99.25±6.72 aCD	32.70±2.05 aC	0.33 bC	0.11±0.01 bC	1.00±0.07 bC	9.42±0.97 aB
	Day 5 FT	43.90±4.34 bC	16.06±1.58 bE	0.37 aB	0.20±0.04 aA	1.27±0.08 aA	6.34±0.92 bBC
HAS	/	/	/	/	/	/	/
POM-HAS	Day 0	44.13±4.61 bD	14.48±1.25 bC	0.33±0.03 aD	0.10±0.02 aD	0.23±0.12a bE	2.16±0.78 aC
	Day 5 RT	52.47±4.74 bEF	14.99±0.76 bD	0.19±0.01 aE	0.12±0.02 aC	0.37±0.02 aD	3.06±0.38 aC
	Day 5 FT	721.53±112.2 1 aC	153.33±22. 02 aBC	0.21 bF	0.06 bcd	0.14±0.06 bD	2.38±1.11 aDE
RS	Day 0	33.48±1.12 bD	6.00±0.11 bDE	0.18 aE	0.28±0.02 aA	0.53±0.07 aCD	1.91±0.12 bC
	Day 5 RT	68.17±2.35 bDE	8.27±0.08 bEF	0.12 bH	0.32±0.02 aA	0.57±0.07 aCD	1.80±0.11 bC
	Day 5 FT	2423.42±320. 23 aB	196.26±21. 48 aB	0.08±0.01 cG	0.04±0.01 bcd	0.16±0.01 bD	4.09±0.82 aCD
POM-RS	Day 0	204.52±7.56 aB	36.20±1.00 bB	0.18. bC	0.31±0.02 aA	0.57±0.02 aCD	1.85±0.05 aC
	Day 5 RT	235.15±29.32 aB	53.14±5.95 abB	0.23 aF	0.27±0.03 abB	0.54±0.05 abCD	2.07±0.41 aC
	Day 5 FT	266.22±56.85 aC	62.38±12.4 0 bDE	0.24±0.01 aDE	0.19±0.05 bC	0.44±0.06 bC	2.49±0.84 aDE
CS	Day 0	128.20±23.23 bC	10.93±3.28 bcd	0.08±0.01 abF	0.19±0.02 aBC	0.20±0.02 aE	1.08±0.02 cC
	Day 5 RT	135.37±22.06 bC	13.05±2.10 bDE	0.10 aI	0.13±0.01 bC	0.20±0.01 aD	1.60±0.17 bC
	Day 5 FT	12244.62±81 1.61 aA	916.53±85. 08 aA	0.08 bG	0.03 cCD	0.15±0.01 bD	4.71±0.27 aBCD
POM-CS	Day 0	382.32±40.66 aA	78.99±5.44 aA	0.21±0.01 bE	0.16±0.01 bC	0.40±0.02 bDE	2.53±0.09 aC
	Day 5 RT	392.58±3.62 aA	79.78±0.71 aA	0.20 bG	0.25±0.03 cB	0.62±0.03 aCD	2.50±0.17 aC
	Day 5 FT	12244.62±81 1.61 aA	916.53±85. 08 aA	0.08 bG	0.03 cCD	0.15±0.01 bD	4.71±0.27 aBCD
WMS	Day 0	6.73±0.48 bD	3.06±±0.19 bE	0.45 aB	0.14±0.03 aCD	5.53±0.03 aA	42.08±9.27 aA
	Day 5 RT	11.85±±0.93 bG	3.63±±0.23 bF	0.31±0.01 bD	0.13±0.01 aC	3.71±0.57 bA	29.51±4.58 aA
	Day 5 FT	510.24±5.56 aC	131.33±6.2 6 aBCD	0.26±0.01 cC	0.02 bD	0.19±0.03 cD	9.31±1.42 bA
POM-WMS	Day 0	12.36±0.27 bD	9.55±0.18 bCDE	0.77±0.03 aA	0.21±0.03 aB	0.31±0.02 aDE	1.48±0.23 bC
	Day 5 RT	18.58±1.71 bFG	8.91±0.80 bDEF	0.48 bA	0.10±0.02 bC	0.45±0.21 aCD	4.27±1.83 aC
	Day 5 FT	211.15±15.02 aC	52.99±3.19 aDE	0.25 cCD	0.08 bBC	0.23±0.03 aD	2.71±0.23 abDE

In addition, G' of POM gel (1 %) and POM fraction that has been separated from POM-HAS that has been thermally treated and centrifuged at 90°C was compared (Fig. 5). G' of centrifuged POM was 10.79 Pa, which is similar to POM gel prepared on its own (13.63 Pa). The slight difference could be due to the gel disturbance by centrifugation or HAS granules affecting POM during centrifugation. Nevertheless, this showed that (1) HAS could be successfully separated from POM even after thermal treatment, and (2) HAS most likely did not exhibit interaction with POM network due to similar G' values of POM obtained through two described ways.

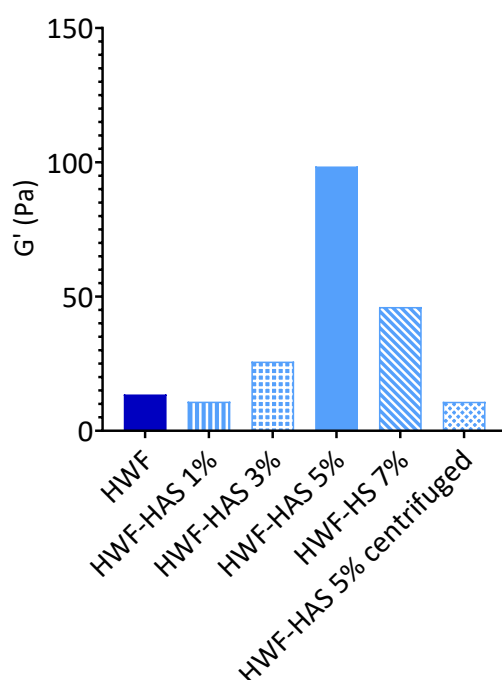


Figure 5. Storage modulus (G') of POM-HAS gels with different HAS concentrations (1-7%) and POM-HAS (5%) after centrifugation.

4. Proposed mechanisms

After characterising POM-starch gels, possible mechanisms which govern their interactions are proposed. The gels have different properties, which are mostly influenced by starch type. A review on starch-hydrocolloid properties published in 2011 (BeMiller, 2011) is still the most detailed review on this topic. One of main takeaways is that in these systems, it is (relatively) easy to obtain results, but the

interpretation is often difficult. Wide propositions of mechanisms of these gels is a valid question – considering various types of starches and NSPs, can the proposed mechanisms be applicable in most of these systems? Here, we try to answer that question: while some of these mechanisms can be applied to all the gels, others are specific for certain starches, depending on their composition, mainly high amylose (HAS) or high amylopectin (WMS) starch.

1) POM – amylose synergistic interactions

Gelatinised starch systems can be described as swollen starch granules dispersed in a continuous amylose phase (Dobosz et al., 2020). When NSP is added to starch gel, increase in G' (and/or viscosity) is higher than the increase of G' if elastic modulus of NSP and starch gel were simply added ($G'(\text{NSP-starch gel}) > G'(\text{starch}) + G'(\text{NSP})$) – this points to formed matrix between starch and NSP, which in literature is widely referred to as synergy between these two molecules (BeMiller, 2011). This is clearly visible in the rheology profile of the gels as well (except POM-WMS) and indicates interactions between NSP and amylose. The exact level of these interactions is hard to prove: some researchers suggest that hydrogen bonds are formed between amylose and NSP (Rong et al., 2022), while other describe entanglements in this system (Liu et al., 2003), and some even proposed that crosslinking is responsible for the viscoelastic profiles of some NSP-starch gels (Lai & Liao, 2002). Some authors have shown that only NSP, amylose, and amylopectin of certain chain length interact with one another (Funami et al., 2005). Nevertheless, this work shows a certain degree of interactions between leached amylose and POM, based on the increase in G' and retaining gel properties after RT and FT storage, and this explains decreased syneresis. Amylose-POM interactions interfere with amylose-amylose interactions, which are the first cause of retrogradation (Dobosz et al., 2020). It is important to highlight that these interactions occur in the continuous phase, which is mainly responsible for viscous properties of NSP-starch gel.

2) Syneresis mechanisms

Syneresis results after RT and FT conditions gave us insights into mechanisms governing interactions between POM, starch and water, and rheology profile of the

gels exposed to different conditions provided more clarity on POM-starch behaviour. G' increased the most in RS and CS after 5 days FT, which can be explained by the highest amount of water expulsion, which in turn increases solids concentration. The increased concentration subsequently leads to higher G' . However, different syneresis mechanisms are likely involved in their behaviour. Fig. 6 shows changes in gel strength and flow transition index (FTI) as well as syneresis.

Phase separation mechanism can be characterised by low gel strength (Fig. 6) and is seen in POM, POM-WMS, and POM-HAS gels under certain conditions. POM-WMS and POM-HAS are strikingly different systems, but the lack of synergistic interactions between starches and POM can be observed in both systems (see chapter 3.4.). In POM-HAS, FT treatment caused more noticeable phase separation as particle jamming system has undergone freezing and thawing. In POM-WMS, RT treatment resulted in the highest syneresis due to antagonistic interactions between POM and WMS. In both cases, phase separation between POM and starches is considered to be the cause of syneresis.

On the contrary, systems with higher gel strength which exhibited higher syneresis can be explained as “poroelastic syneresis”. Such systems (RS and CS) can be described as porous solid materials containing liquid phase in their pores – i.e. water in starch ghosts. When subjecting RS and CS to freezing and thawing, changes in water phase volume also influence porous structure of the gels. Therefore, when applying stress (as in amplitude sweep) on porous materials, the volume fraction of the pores is affected and material deforms elastically. This can be seen in significant increase of G' (Table 1) and gel strength (Fig. 6) in RS and CS after FT treatment. When POM is added to RS and CS, it is clear that syneresis is lower and that gel strength and flow transition index remain similar when undergoing syneresis protocols, as they can be seen clustered together (Fig. 6). POM maintains gel structure and protects against water separation.

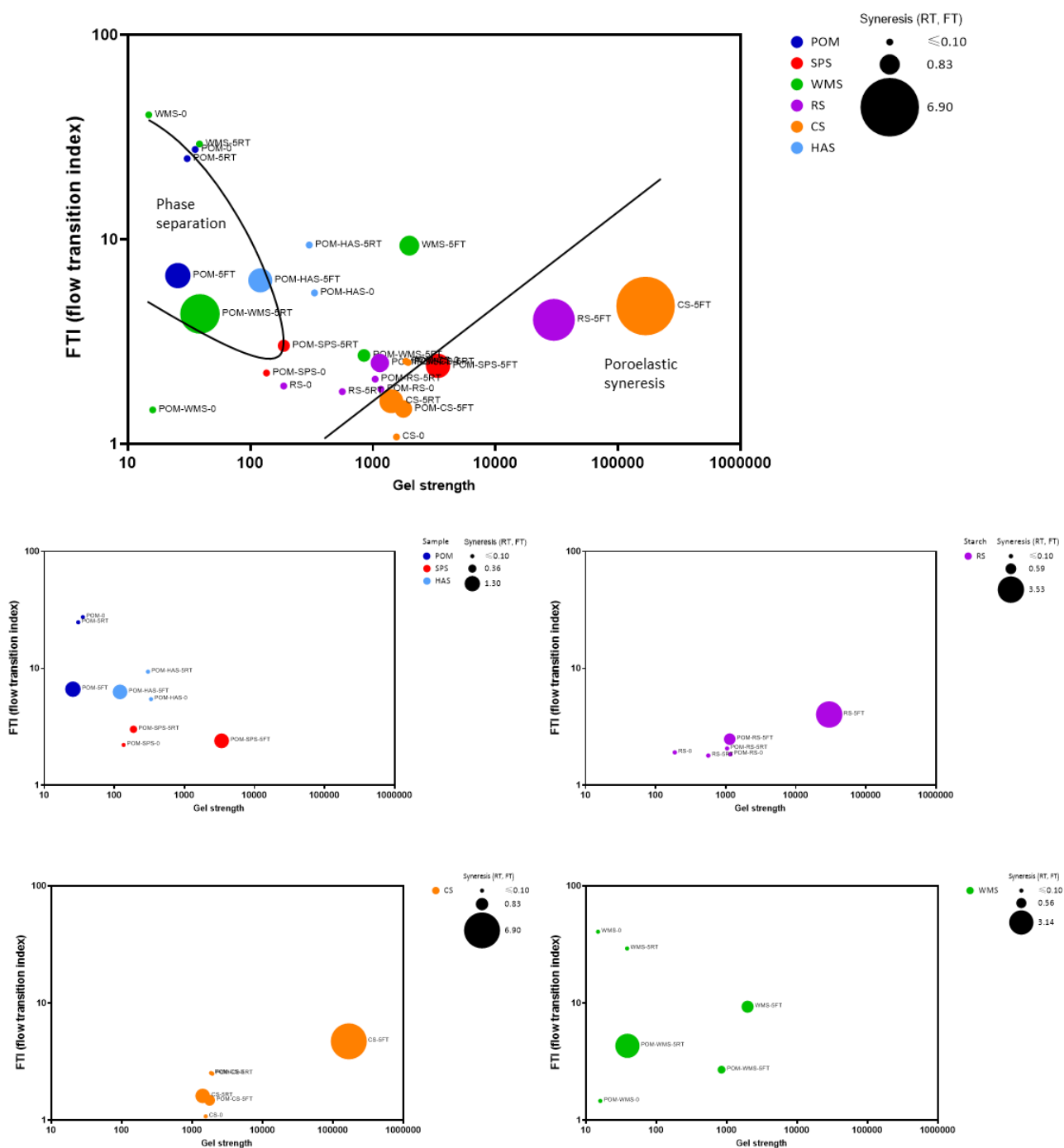


Figure 6. Gel strength (expressed as $G'/\tan \delta$) and flow transition index (FTI) of all gels. Size of circle of a gel corresponds to syneresis. Four smaller panels below represent the same image, but gels are separated for easier guidance. This representation of rheological properties and syneresis allows for mechanisms hypothesis to be formed: gels that have low gel strength, but high FTI exhibit phase separation, while gels mechanism that have high strength and relatively low FTI can be explained by poroelastic syneresis.

3) Unique system of HAS-POM gel

High amylose starches have a higher gelatinisation temperature when compared to their conventional or waxy starch counterparts (Li et al., 2019). For complete gelatinization, often autoclaving or longer hydrothermal treatment is required. Freitas *et al.* (2003) have described rheological properties of galactoxyloglucan from *Hymenaea courbaril* mixed with high amylose corn starch (66% amylose) and prepared by heating the fibre-starch mixture at 96 °C for 30 minutes. Their results showed interaction between amylose and galactoxyloglucan, which can be seen in increased thermal stability and viscosity of the gel. On the other hand, Xie *et al.* (2020) have shown using that tamarind seed gum facilitates effective bonding with amylopectin rather than amylose (treatment at 121 °C for 1 h was used for high amylose starch, while normal and waxy maize starches were gelatinised at 95 °C for 30 min).

In this work, the hydrothermal treatment for all 5 gels are the same, and it can be assumed that HAS did not fully gelatinise. Moreover, fluorescent microscopy (Fig. 3) and expanded rheology profiles (Fig. 5) of POM-HAS gels support the theory of particle (HAS) filled gel (POM) which is different from other gelatinised gels. Therefore, the POM-HAS gel has a unique system with starch granules when they are not fully gelatinised.

4) Competition for space

As is known, amylose and amylopectin have very different structure and functionality. Amylose is a helical molecule with molecular weight of 10^6 Da, while amylopectin is highly branched and is one of the largest biopolymers (molecular weight is 10^8 Da) (Li et al., 2019). On average, starch has 25% amylose and 75 % amylopectin. High amylopectin or waxy starches are made of 99% amylopectin, which gives them distinctive properties when compared to traditional starches. They usually have higher swelling and water holding capacity, lower rate of retrogradation, and decreased syneresis when compared to their conventional counterparts (Hsieh et al., 2019). During retrogradation, amylopectin forms a network via amylopectin sidechains within the same molecule interacting with each other. However, when

NSP is added, it disturbs potential amylopectin network since NSP forms matrix on its own. Moreover, interactions between same type of molecules are favoured over interactions between different types of molecules (Choi & Yoo, 2008).

NSP and amylopectin do not interact with each other in a way which NSP and amylose might interact (and result in higher viscoelastic moduli). Therefore, another type of mechanism is proposed, specific for amylopectin and NSPs (in this case POM): competition for space. This mechanism refers to antagonistic behaviour between amylopectin and POM and highlights their irregular and disjointed matrix which affects syneresis (RT syneresis was lower in WMS than in POM-WMS, the only example of this trend) and unchanged viscoelastic moduli (oscillatory rheology showed unchanged storage modulus when POM was added to WMS gel, confirming the lack of interactions between these two gel constituents). Overall, competition for space between amylopectin and POM might cause unwanted functional and sensory characteristics in products containing waxy starch which are stored at room temperature, but POM nevertheless showed reduction of FT syneresis, therefore POM could be useful for cold storage food products.

5) Other possible mechanisms

Both POM and starch are hydrophilic biomolecules. POM has a high water retention (Phan et al., 2020), and, during gelatinization, starch requires water to transform from crystalline to disordered state. In composite system, NSP reduces the volume fraction of available water, and decreases gelatinization of starch (Khanna & Tester, 2006). This is especially true for NSPs which create gel and network matrices (such as POM). When adding psyllium to different starches, it has been shown that psyllium influenced thermal properties, swelling power, and gelatinization of wheat, potato, and tapioca starches (Sevilmis & Sensoy, 2022). Addition of psyllium decreased gelatinization enthalpy at different water contents (solids:water = 1:1, 1:2, 1:5), and authors explained this occurrence with competition for water mechanism. However, it should be highlighted that addition of psyllium in said work was 50%, which is significantly higher than amount of fibre usually added to starch gels or to starch containing foods, thus the extent of this effect should be explored with varying concentrations of fibre.

Besides the changes in gelatinization, competition for water could explain the increase in G' of POM-starch gels when compared to $G'(\text{POM}) + G'(\text{starch})$, as well. Both of these carbohydrates require aqueous environment to exhibit gelling properties, thus they are able to interact with each other and with water molecules to establish a weak gel. Moreover, significant decrease in syneresis of composite gels when compared to starch gels after FT treatment indicates that POM reabsorbed released water from starch. To the best of our knowledge, there are no papers that compare the influence of NSP added in starch gels at room temperature and freeze-thaw syneresis. We propose that both protocols are important for uncovering mechanisms which govern starch-hydrocolloid-water interactions.

Besides competition for water theory, it is important to mention possible structural mechanism which influences functionality and properties of POM-starch gel, which is that POM forms occlusive film around starch granules. In other work, POM was described as barrier around starch granules which caused restriction of swelling and gelatinization (Atac & Sensoy, 2022). This envelopment of starch granules by is characteristic for highly viscous gel (such as POM), and could lead to reduced syneresis, and lower digestibility.

Both of mechanisms explained above are probable for our POM-starch systems as well, however, we have decided to focus on primarily rheological characterization of said gels, and proof for these mechanisms would require additional testing, such as differential scanning calorimetry, which is planned for future work.

5. Conclusion

Combining a specific fraction from *P. ovata* mucilage (heteroxylan rich fraction extracted at 65 °C) with 5 different starches, gave us an insight into intermolecular behaviour between two complex polysaccharides. In all cases, POM had a protective role against freeze-thaw syneresis, and it maintained elastic properties after freeze-thaw treatments, most notably in POM-corn starch gel and POM-rice starch gels. Depending on amylose to amylopectin ratio, POM-starch gels displayed distinct rheology behaviour – it can be concluded that POM and amylopectin had antagonistic

behaviour, which is characterised by competition for space, while POM-amylose displayed synergistic behaviour. These occurrences were confirmed by microscopy, too, as structure of POM-starch gels greatly varied. Overall, when developing a starch rich food product, POM can have a protective effect, but certain prerequisites should be met, such as type of starch and storage conditions.

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CHAPTER 6

Fibre fractions from *Plantago ovata* mucilage show contrasting effects on structure and digestibility of corn starch with varying amylose content at different temperature treatments

Title:

Fibre fractions from *Plantago ovata* mucilage show contrasting effects on structure and digestibility of corn starch with varying amylose content at different temperature treatments

Authors:

Lucija Strkalj^{1,2}, Gleb E. Yakubov², Rachel A. Burton¹, James M. Cowley^{1*}

¹School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

²Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom

*Corresponding author

Highlights:

- Two psyllium fibre fractions show different properties on pasting of corn starches.
- F1 increased starch hydrolysis in all gels; F2 showed decrease or no effect.
- Fibre fractions from same source show contrasting functional effects on corn starch.

Abstract:

Psyllium, a potent dietary fibre derived from *Plantago ovata* seeds, is frequently used as a food additive due to its remarkable gelling, water uptake capacity, and texturizing properties. Structurally, psyllium fibre is an intricate and densely branched heteroxylan which, depending on extraction conditions, has shown extremely diverse rheological and functional properties. Even though psyllium rheology is well studied, there is a lack of understanding on how these fractions can be exploited for food improvement. Therefore, we isolated two psyllium fractions (F1 and F2) and

fabricated starch-fibre gels using starches with differing amylose content. To fully understand mechanisms between fractions and starches, we applied three different temperature treatments (95, 120 and 140 °C) by utilising high temperature rapid visco analysis (HT-RVA), resulting in 27 starch and starch-fibre gels. HT-RVA graphs have shown specific peaks and viscosity changes for certain fibre-starch combinations, pointing to differences in interactions between amylose and F1 and F2. Texture properties of the gels were mostly influenced by temperature treatments, but amylose leaching and starch hydrolysis by α -amylase were significantly changed by fibre addition, leading to an interaction between fibre and temperature. F1 caused an increase in estimated glycaemic index, but F2 reduced it. SEM images have shown changes in gel microstructure depending on fibre addition and temperature potentially related to phase separations and fibre melting. This work highlights that fibre fractions from the same source can have contrasting effects on functional and health properties of starch-based food systems, which may be highly valuable tools for developing healthy products especially if their recombination was optimised.

Keywords:

Psyllium, corn starches with varying amylose, pasting profile, amylose leaching, starch hydrolysis

Abbreviations:

CS = corn starch, eGI = estimated glycaemic index, HACS = high amylose corn starch, HT-RVA = high temperature rapid visco analysis, k = hydrolysis rate, Mw = molecular weight, PT = pasting temperature, RDS = rapidly digestible starch, RS = resistant starch, SDS = slowly digestible starch, WCS = waxy corn starch, WRC = water retention capacity, WSC = water swelling capacity

1. Introduction

Starch is estimated to have the largest contribution to worldwide energy intake (Burrell, 2003; Jebb, 2015; Svihus & Hervik, 2016). Besides starch-rich staples like bread, rice, and pasta, starch is widely implemented as an ingredient into array of food products due to the large number of source options, associated variation in functionalities, and the ease of application. When assessing the impact of ingredients and processing on techno-functional properties, starch gels are often used in research and industry, as they are mostly inexpensive, can be used for many experiments and in general, reflect in-food behaviours (BeMiller, 2011). Producing starch gels is usually straightforward: mixing starch with water, heating until reaching gelatinization followed by achieving gel-like consistency upon cooling. Starch properties after cooking determine the quality of final product, such as viscosity, digestibility, and storage stability. While starch has a pivotal role in food production and nutrition research, it is essential to recognize its disadvantages, as well. Storage stability can be challenging, as starch gels can be prone to syneresis under lower temperatures, which influences sensory perception and product structure (Srichuwong et al., 2012; Takeiti et al., 2007). Moreover, starchy foods can have a high glycaemic index, which leads to spike in blood glucose levels, which in turn can lead to metabolic disease (Lal et al., 2021).

The addition of non-starch polysaccharides (NSP), such as fibres and gums can improve structure and texture, storage stability, and digestibility of starch gels. Generally, NSPs delay starch gelatinization and reduce digestibility through different mechanisms. NSPs can form a barrier around starch granules and restrict amylases from reaching the substrate, they can compete for water and thus reduce starch swelling and gelatinization, and they can non-competitively bind to α -amylase (Li, 2022). Addition of pectin from citrus peel (rhamnogalacturonan-I) to potato starch decreased hardness of the gel and increased its slowly digestible starch content (Yin et al., 2021). Heteroxylan from corn fibre decreased retrogradation and led to more stable corn and wheat starch gels (Qiu et al., 2016). Wheat arabinoxylan delayed long-term retrogradation and increased hardness when added to wheat starch gel (Yan et al., 2022). It has also been shown that certain types of pectin with low degree of

esterification and hydrodynamic radius decreased maize starch digestibility by non-competitive inhibition of α -amylase (Bai et al., 2021). Overall, research has shown that NSP addition can have a pronounced effect on the quality of starch gels and the explanatory interactions and mechanisms can be different (BeMiller, 2011).

A source of NSP fibre that has been of particular interest to our research groups is *Plantago ovata* Forssk., widely known as psyllium (Cowley & Burton, 2021; Phan et al., 2020; Yu et al., 2017). Psyllium husk is produced from the milled coating of *P. ovata* seeds which, when in contact with water swells and forms a strong gel with complex structure (Cowley & Burton, 2021; Yu et al., 2017). It is widely used in food industry, as an addition to conventional bakery products, gluten-free bread, yoghurts, jams, and can be used as fat replacer, stabiliser, and texturiser (Belorio & Gómez, 2022). It has numerous health benefits, such as cholesterol reduction, satiety and appetite regulation, gastrointestinal relief, and lower glycaemic index when incorporated into starchy products (McRorie et al., 2021). When incorporated into wheat, potato, or tapioca starch gels, psyllium can reduce rapidly digested starch and increase slowly digested starch (Sevilmis & Sensoy, 2022). Psyllium with whey protein isolate can improve solubility, tensile strength, and elongation of lotus rhizome starch film, which is useful for biodegradable packaging (Sukhija et al., 2018). When comparing psyllium and xanthan gum properties in maize starch, it was reported that both NSPs reduced hardness, while psyllium changed pasting properties (Belorio et al., 2020). Due to interest in *P. ovata* fibre gel structure and its high complexity, different extraction protocols have been developed (Cowley et al., 2021; Ren, Yakubov, et al., 2020; Yu et al., 2017; Zhou et al., 2020). It was confirmed that fractions extracted with H₂O at lower temperature (up to 25 °C) forms viscoelastic fluid and consists of heteroxylan with a minor (>10%) pectin component. Fractions subsequently extracted at higher temperatures (65 °C and higher), with alkaline solvents (2M KOH), or with intense shaking, were found to consist of highly branched heteroxylan (depleted of pectin) which forms significantly stronger gels (Cowley et al., 2021; Ren, Yakubov, et al., 2020; Yu et al., 2017, 2019, 2021). Even though detailed rheology and structure studies of different fractions have helped elucidate complex structure and behaviour of psyllium gels, there is a lack of practical application of said fractions. Moreover, there is a gap

in understanding how *P. ovata* fibre and particularly fractionated fibre differentially affect starch gels and products. For example, it is the third most used addition in gluten-free breads (Belorio & Gomez, 2021). Therefore, this complex gel represents an opportunity to research the impact of distinct fibres in starch systems.

In this work, a straightforward extraction method was used to obtain two different fibre fractions from the same source (commercial psyllium husk) which had differing functional properties. The work in this chapter is an expansion of work shown in the previous chapters, and is aimed to be more commercially relevant. The chosen material is psyllium husk, which is already widely used as an ingredient for improving structure, nutrition, and functionality of food products. Whole *P. ovata* seeds can be purchased in retail stores, but are harder to find and are less likely to be used as a starting material for fibre extraction. Next, the fractionation method was streamlined and consisted of only one mixing step, yielding two fractions. The method is simplified and can be adjusted to a pilot plant or a commercial kitchen. The practical application of said fibres was then assessed on three corn starches with varying content of amylose under three different temperature conditions by studying techno-functional properties, structure, and digestibility of fibre-starch gels. In this study, we prioritized examining the outcomes over elucidating the underlying mechanisms for the aim of studying how distinct fibre additions and processing conditions affect different starches, and how this knowledge can be translated into digestibility and formulation of new food products. Nevertheless, results shown here provide a basis for continuing further study of interaction and mechanisms between starch and *Plantago* fibre fractions.

2. Materials and methods

2.1. Materials

Three types of starch were used: corn starch (Sigma Aldrich, S4126), waxy maize starch (VPA, Queensland), and high amylose starch (Hylon VII, Ingredion, New South Wales). For starches, we use following abbreviations: CS = corn starch, WCS = waxy corn starch, HACS = high amylose corn starch.

Psyllium husk (Bonvit, Woolworths, South Australia) was used for extraction of two fibre fractions. 8 g of husk was dispersed in 800 mL RO H₂O and stirred (400 rpm) for 4 h at 4 °C. The mixture was centrifuged at 3600 rpm (30 min, 4 °C) (5810R centrifuge, Eppendorf, New South Wales). Upper layer (Fraction 1) was separated from lower part (Fraction 2). Upper fraction (supernatant) was slightly viscous liquid, while lower fraction (pellet) was gel with solid husk remnants (visually determined). Both fractions were separately dried overnight in an air oven at 95 °C. After drying, fibres were ground in coffee grinder or milled in a mixer mill (Retsch MM400, Germany) (75% filling, 10 min, 30 Hz), and sieved using 200 µm sieve. For fibre fractions we use following abbreviations: F1 = dried and sieved supernatant, F2 = dried and sieved pellet.

Porcine pancreatic α -amylase (E-PANAA) was purchased from Megazyme (Neogen Australasia, Queensland, Australia). Amylose from potato (A0512), D-maltose monohydrate (63418), 4-hydroxybenzhydrazide (H9882), trichloroacetic acid (T0699), sodium carbonate (223530), iodine (207772), and sodium phosphate monobasic (S0751) were purchased from Sigma Aldrich (New South Wales, Australia). Sodium phosphate dibasic (SA026), potassium iodide (PA001), and sodium hydroxide (SA000) were purchased from ChemSupply (South Australia). Hydrochloric acid (RP1104) was purchased from RCI Labscan (Victoria). Amylopectin (A0456) from waxy corn was purchased from Chem-Supply (Gillman, South Australia). All used chemicals were of analytical grade.

2.2. Methods

2.2.1. Characterization of starches and fibres

Apparent amylose content of starches was determined by 96-well plate assay developed by Kaufman et al. (2015) without any modifications.

Particle size distribution was carried out using a multi-laser Particle Size Analyser (PSA1190, Anton Paar, Austria) using a dispersion air setting of 4000 mbar. The internal Kalliope software calculated the particle size distribution on a volume basis. Results are means of duplicate runs.

Fibre yield was determined by weighing the mass of psyllium before extraction process, and weighing the masses of F1 and F2 after drying (Equation 1).

Equation 1:

$$\text{Fibre yield (\%)} = \frac{\text{mass of extracted fibre (g)}}{\text{mass of psyllium husk used for extraction (g)}} \times 100$$

FTIR spectra of powdered F1 and F2 were collected using a Nicolet iS20 FTIR spectrometer (Thermo Scientific, USA) using 48 scans at a resolution of 4 cm.

Monosaccharide composition of F1 and F2 was determined by using reverse phase high performance liquid chromatography (RP-HPLC) of 1-phenyl-3-methyl-5-pyranoside (PMP) derivatives following the process described in Hassan et al. (2017) with modifications specified in Cowley et al. (2021) where the fibre was pre-dispersed at 1 mg/mL in hot water. Area under the peaks was compared to standard curves of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose and fucose.

Water retention capacity (WRC) and water swelling capacity (WSC) of F1 and F2 were assessed according to method by Noguerol et al. (2022) with no modifications.

2.2.2. Pasting behaviour

A Rapid Visco Analyser (RVA 4800, Perten Instruments, New South Wales) was used to simultaneously prepare and assess the pasting behaviour of starch and starch-fibre gels. An amount of 1.875 g (dw) of starch was weighed into the aluminium RVA canister and H₂O was added to a total mass was 25 g. When preparing starch-fibre gels, 0.1 g of fibre was added, so the final mass was 25.1 g. Addition of fibre based on the total gel mass was 0.4%, while based on starch mass was 5.3%.

Three RVA protocols were used based on maximum temperature ($T_{\max}$) reached during pasting: 95, 120, and 140 °C. To ensure all samples were treated at corresponding $T_{\max}$ for equal time period and rate of heating/cooling was equal (12.3 °C/min), protocols had varying time lengths. Therefore, all protocols followed the same outline (hold at 25 °C for 2 min → increase temperature to $T_{\max}$ → hold at $T_{\max}$ for 2.5 min → decrease temperature back to 25 °C → 25 °C hold at for 2 min), but the

lengths of the runs were: $T_{\max}$ 95 °C – 18.10 min, $T_{\max}$ 120 °C – 22.20 min, and $T_{\max}$ 140 °C – 25.40 min. Paddle speed was 960 rpm during the first minute, and 160 rpm during the rest of the protocol.

Gels from the RVA were then used for following experiments (described below). In Table 1, we specify the sample preparation and abbreviations which will be used throughout the rest of this work.

Table 1. List of starches at fibres treated at specified temperature to prepare gels and corresponding sample name.

Starch	Fibre	Temperature treatment (°C)	Sample name
Corn starch	/	95	CS-95
Corn starch	F1	95	CS-F1-95
Corn starch	F2	95	CS-F2-95
Corn starch	/	120	CS-120
Corn starch	F1	120	CS-F1-120
Corn starch	F2	120	CS-F2-120
Corn starch	/	140	CS-140
Corn starch	F1	140	CS-F1-140
Corn starch	F2	140	CS-F2-140
Waxy corn starch	/	95	WCS-95
Waxy corn starch	F1	95	WCS-F1-95
Waxy corn starch	F2	95	WCS-F2-95
Waxy corn starch	/	120	WCS-120
Waxy corn starch	F1	120	WCS-F1-120
Waxy corn starch	F2	120	WCS-F2-120
Waxy corn starch	/	140	WCS-140
Waxy corn starch	F1	140	WCS-F1-140
Waxy corn starch	F2	140	WCS-F2-140
High amylose corn starch	/	95	HACS-95
High amylose corn starch	F1	95	HACS-F1-95
High amylose corn starch	F2	95	HACS-F2-95
High amylose corn starch	/	120	HACS-120
High amylose corn starch	F1	120	HACS-F1-120
High amylose corn starch	F2	120	HACS-F2-120
High amylose corn starch	/	140	HACS-140
High amylose corn starch	F1	140	HACS-F1-140
High amylose corn starch	F2	140	HACS-F2-140

2.2.3. Texture profile analysis

An aliquot of 4.5 mL of RVA gels were taken and placed into 5 mL plastic cylindrical tubes. TA.XTplusC Stable MicroSystems texture analyser (Key Diagnostics Pty Ltd, New South Wales) with a small rounded cylindrical probe (4 mm radius, stainless steel with radius end, P/MT, Stable Micro Systems, UK). Triggering force was 1 g, pre- mid- and post-test speeds were 20 mm/s, penetrating to the sample depth of 15 mm (50% of

total height) after which the probe returned to original position. Exponent Connect version 8, 0, 17, 0 programme was used for data collection, and hardness and adhesiveness were calculated.

2.2.4. Freeze-thaw syneresis

Freeze-thaw syneresis of RVA gels was done according to Ma et al. (2019) with some modifications. Samples were subjected to freeze-thawing cycles for 15 days (22h at -20 °C, followed by 2h at 40 °C), after which the upper water layer was carefully removed without centrifugation. Syneresis was calculated as percentage of weight of gel after syneresis divided by weight of gel on day 0 (equation 2).

Equation 2:

$$\text{Syneresis (\%)} = \frac{\text{weight of gel after removing water layer (g)}}{\text{initial weight of the gel (g)}} * 100$$

2.2.5. Scanning electron microscopy (SEM)

A thin layer of gel samples from RVA were immediately smeared onto SEM stubs with carbon stickers and then rapidly frozen at -80 °C freezer. They were then freeze dried at -40 °C for 48 h. Freeze-dried samples were sputter-coated with gold. Scanning electron microscope FlexSEM 1000 (Hitachi, Japan) was used in high-vacuum mode with spot size of 10.0 kV voltage, and FlexSEM 1000 software was used to obtain images. At least three locations were randomly chosen to take micrographs of each sample, and magnification of CS and HACS was 250x, and for WCS it was 150x.

2.2.6. Determination of leached amylose

Amylose leaching assay was adapted from Ma et al. (2019). Five mL of RVA gels were mixed with 5 mL water and shaken for 2 min (without any ball bearings, 30 Hz) using a mixer mill (MM400, Retsch, Germany). After shaking, the gel was centrifuged for 30 min at 16 000 g and 25 °C (Eppendorf 5417R, Germany). Three mL of supernatant was taken and mixed with 6 mL of 0.33M NaOH, followed by heating at 95 °C for 30 min. After the mixture was left to cool to room temperature, 100 µL was taken and mixed with 5 mL of 0.5% TCA and 50 µL of 0.05N I₂-KI dye. After brief vortexing, mixture was

left in the dark to allow for colour development. After 30 min, 300 μ L of the solutions was pipetted into a well in 96-well plate with flat bottom. A standard curve was prepared following the same process with a known concentration of amylose. Absorbance was read using plate reader (μ Quant, Bio-tek instruments, United States) at 620 nm.

2.2.7. Starch hydrolysis by α -amylase and maltose quantification by PAHBAH assay

Starch digestibility by α -amylase was determined based on Edwards et al.'s work (2019). Starch to α -amylase ratio is kept constant in all samples, meaning that 100 mg of starch (in starch gel or in starch-fibre gel) was placed into 15 mL tubes and suspended in 10 mL phosphate buffered saline (pH=7.4). After brief homogenising (PROScientific Premium MaX homogenizer, PRO Scientific Inc., CT, USA) sample tubes were placed in rotary tube mixer inside an incubator (Bioline, Edwards Instrument Company Australia, New South Wales) at 37 °C and equilibrated for 30 min. Before starting the reaction, 100 μ L of each sample was transferred into 1.5 mL microcentrifuge tube which contained 100 μ L of 0.3 M Na_2CO_3 ("stop solution"). The purpose of stop solution is to inactivate the enzyme and stop the reaction at each time point. To start the enzymatic reaction, porcine pancreatic α -amylase was added. Prior to that, the enzyme was carefully weighed so the enzyme activity was 4.5 AU/mL, and dissolved in PBS. Preliminary experiments were carried out to determine the activity, and 4.5 AU/mL was chosen. One activity unit is defined as the amount of α -amylase needed to liberate 1.0 mg of maltose from starch in 3 min at pH=6.9 at 20 °C. Immediately after the addition of the enzyme, the tubes were returned into the incubator and rotated at 37 °C for the rest of the experiment. At pre-defined time points (2, 5, 7, 10, 15, 30, 90, 120, 150 and 180 min) 100 μ L aliquots were taken from the reaction solution and placed into microcentrifuge tubes with equal amount of stop solution. After brief vortexing, all tubes were centrifuged at 15 000 g for 5 min (Eppendorf 5417R, Germany) to exclude any starch remains and 100 μ L of supernatant was placed into new microcentrifuge tubes, which were then used to determine the amount of reducing sugars.

Reducing sugar analysis (PAHBAH assay) was used to determine the amount of reducing sugars which are the product of α -amylase reaction at each sampled time point (Lever, 1972). Supernatants were diluted (1:20) in water, and 100 μ L of the diluted samples was placed in heat-safe microcentrifuge tube which contained 1 mL of PAHBAH reagent (preparation: 250 mg of p-hydroxybenzoic acid hydrazide dissolved in 4.75 mL of 0.5 M HCl and 45 mL of 0.5 M NaOH). The mixture is vortexed, and then placed in boiling water bath (100 °C) for 5 min. After cooling to room temperature (15 min), 300 μ L of each solution was transferred to a well in flat bottomed 96-well plate with and the absorbance was measured at 405 nm (μ Quant, Bio-tek instruments, Queensland). Standards containing a known concentration of maltose (concentrations ranging from 100 μ M to 1000 μ M) were prepared the same way as described above and used for creating the calibration curve against which the samples were measured. Absorbance of fibre (1% w/v in PBS) was tested using PAHBAH to ensure there is no absorbance which would affect the maltose measurement.

2.2.8. Statistical analysis

Data obtained from described experiments is presented as mean and standard deviation of at least triplicates. Starch hydrolysis curves were plotted in OriginPro 2022b software and fitted to first-order equation using non-linear regression, with parameter “a” as the extent of hydrolysis (C_{∞}) and parameter “b” as the rate of hydrolysis (constant k). Area under curve (AUC) was calculated according to Goni et al. (1997). Two-way analysis of variance (ANOVA) with post-hoc Tukey test (0.05 level of significance) was performed in OriginPro 2022b software. Graphs were made in GraphPad Prism 9.0.0. software, unless otherwise stated.

3. Results and discussion

3.1. Characterization of starches and fibres

Starch particle size and amylose content can be found in Fig. 1A. CS and HACS had similar granule size (14.639 and 12.014 μ m, respectively), while WCS showed larger

granules (40.034 μm). HACS had the highest amylose content (63.1%), while WCS had the lowest (5.9%), as expected. Amylose content of CS was 29.2%, which agrees with the published literature (Chung et al., 2015).

Properties determined for fibres were yield, water retention capacity, water swelling capacity (Fig. 1b), arabinose to xylose ratio (A:X), and pectin to arabinoxylan ratio (Fig. Dd), monosaccharide composition (Fig. 1C), and FTIR spectra (Fig. 1E). Both fibres were heteroxylans, but F1 contained 10% pectin (calculated as rhamnose + galacturonic acid). F2 had a higher A:X ratio than F1 (0.36 and 0.17, respectively), which indicates higher degree of branching. Yu et al. (2017) have extracted 3 fractions of *P. ovata* fibre (25, 65 °C and alkaline extraction) and reported similar monosaccharide composition to ours (F1 similar to fraction extracted at 25 °C, and F2 similar to a combination of the 65 °C and alkaline fraction). They also reported decreased extractability and increasing gelling capacity with further extraction steps (Yu et al., 2017). F1 had much lower yield than F2 (21.9% and 72.4%, respectively). Cowley et al. (2020) have studied three-step extraction of *P. ovata* mucilage – extraction at 25 °C followed by 65 °C, and lastly, extraction via intense shaking. They reported that the fraction at extracted 25 °C accounted for 31% of extracted mucilage (Cowley et al., 2020). F1 had slightly lower yield as it was extracted at 4°C (21.9% as opposed to 31% in Cowley et al.'s work), however they do seem comparable. F2 had a higher water retention capacity (WRC) and water swelling capacity (WSC) (Fig. 1B). WRC of F1 was 12.03 g water/g fibre, while WRC of F2 was 28.42 g water/g fibre. This shows that F2 could retain higher amount of water, which leads to increased swelling. Consequently, WSC of F1 was 5.27 mL water/g, and of F2 was 9.67 mL water/g. Noguero et al. (2022) have reported WRC and WSC of whole psyllium husk which were 25.8 g water/g dry sample and 11.7 mL water/g dry sample, respectively. Our WRC and WSC values were somewhat higher, which is most likely due to the fractionation and our fractions being in powder form as powders can absorb more water. It has been reported that psyllium husk powder had water holding capacity of 45.715 g water/g psyllium powder (Kale et al., 2016). The FTIR spectra of F1 and F2 are very similar and is typical for heteroxylan fibre, as expected (Fig. 1E). Broad peak from 3600 to 3000 cm^{-1} represents O-H stretching, followed by smaller peak at ~ 2900

cm⁻¹ of C-H stretching (Ren, Yakubov, et al., 2020; Yu et al., 2017). A slight difference can be seen in the peak around 1600-1700 cm⁻¹ where F1 showed higher are more pronounced peak. This can be attributed to C=O stretching of the carboxyl group in galacturonic acid (Ren, Yakubov, et al., 2020). This agrees with monosaccharide composition which showed that F1 contains galacturonic acid. The cluster of peaks from 1550 to 1100 cm⁻¹ can be explained by C-O-C glycosidic bonds vibrations and C-OH stretching (Ren, Yakubov, et al., 2020). FTIR spectra can also shed light on the chemistry of heteroxylan, as evidenced by Ren et al. (2020). These authors found that the ratio between peaks at ~978 cm and ~955 cm in the second-derivative spectra, were correlated with A:X ratios determined by HPLC and NMR (higher ratios correlated with higher A:X). While these peaks were present at slightly different wavenumbers, we found the opposite (Fig. 1d, inset), though we only had two fractions and F2 contained residue which differed from Ren et al.'s work. In other work by Robert et al. (2005), when wheat endosperm polymers were studied with FTIR, AXs that were more highly substituted had greater peaks at ~955 cm⁻¹ and lower peaks at 985 cm⁻¹. This is consistent with our results as F2 is expected to be more highly substituted based on previous work and its A:X is greater (0.36). Overall, the FTIR results support the monosaccharide analysis performed here (loss of pectin and more complex substitution in F2 vs F1) and previous findings.

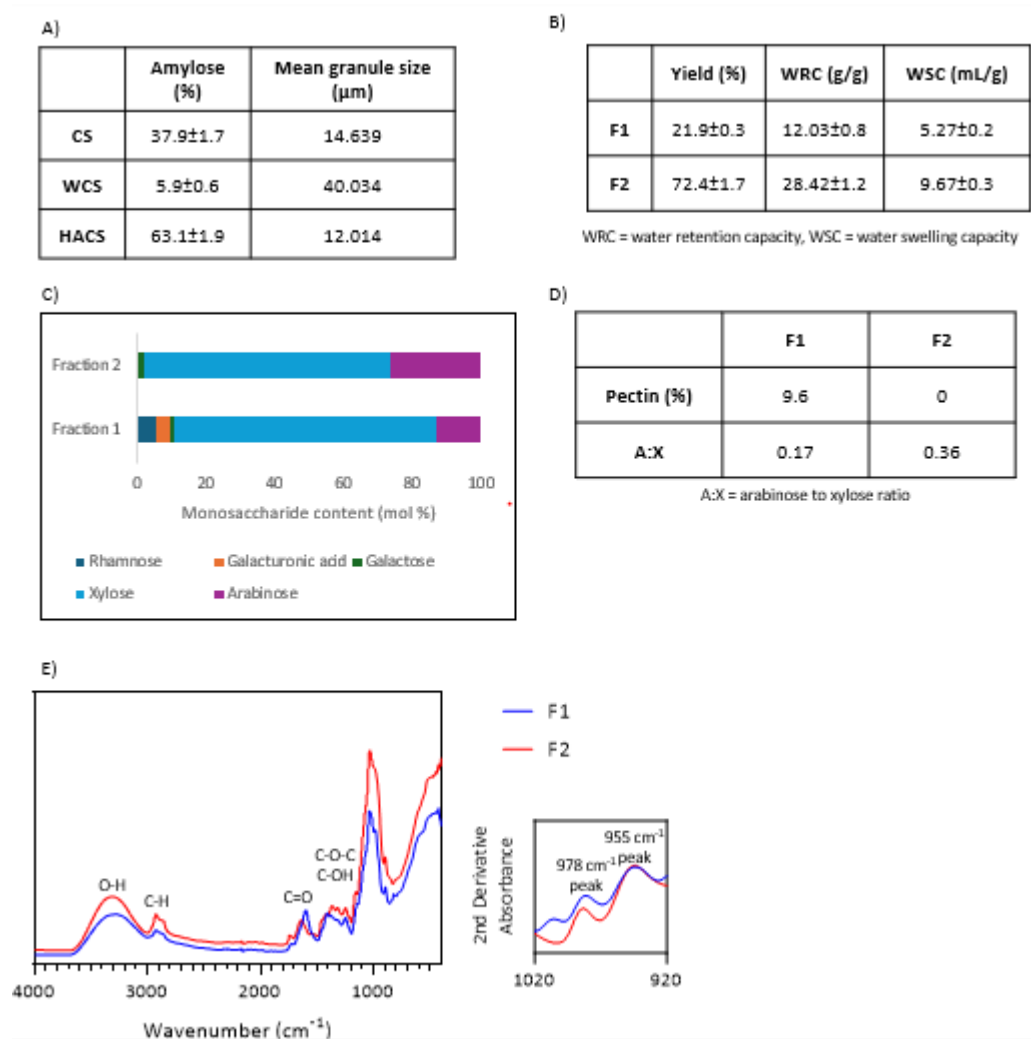


Figure 1. Properties of starches (1A – amylose content and mean granule size) and fibres (1B – yield, water retention capacity, and water swelling capacity; 1C – monosaccharide composition, 1D – pectin content and arabinose to xylose ratio), 1E – FTIR spectra with 2nd derivative inset). All measurements are taken as triplicates at least, unless otherwise stated.

3.2. RVA profiles

RVA provides insight into the cooking behaviour of starches, as starch and starch-fibre mixed with H₂O are subjected to a heating-holding-cooling-holding cycle (Balet et al., 2019). RVA pasting profiles display changes in viscosity as starch granules swell,

gelatinise, rupture and leach amylose, lose crystalline regions and, finally, as amylose chains reassociate due to the lowering of temperature.

Before discussing results, a clarification should be made. Works reporting on starch properties and the use of RVA oftentimes use the terms “gel” and “paste” interchangeably. However, there are certain differences. The term “paste” usually refers to the hot paste from RVA, while the term “gel” refers to the cooled (and sometimes stored) paste which has gone through or is going through the process of retrogradation (BeMiller, 2011; Ren et al., 2023). Paste is a viscoelastic fluid which does not stand on its own, while gel can self-support its structure. In our work, RVA profile ended at 25° C, thus producing samples at room temperature. Moreover, all samples except WCS (all temperatures), WCS-F1 (all temperatures) and HACS at 95 °C (with and without fibre) could support itself (not shown). WCS and WCS-F1 samples are technically pastes, and HACS samples are dispersions of starch and starch-fibre in H₂O. However, for the ease of reading, we use the term “gel” in the remainder of the paper.

1) Influence of temperature on pasting characteristics of the three corn starch gels with varying amylose content

RVA profiles of all gels are presented in Fig. 2. Previous papers (Liu et al., 2019; Tian et al., 2022) have been published on HT-RVA treatment on corn starches with varying amylose content. However, to understand the effect of F1 and F2 on starches, we include their pasting curves as benchmarks for comparison.

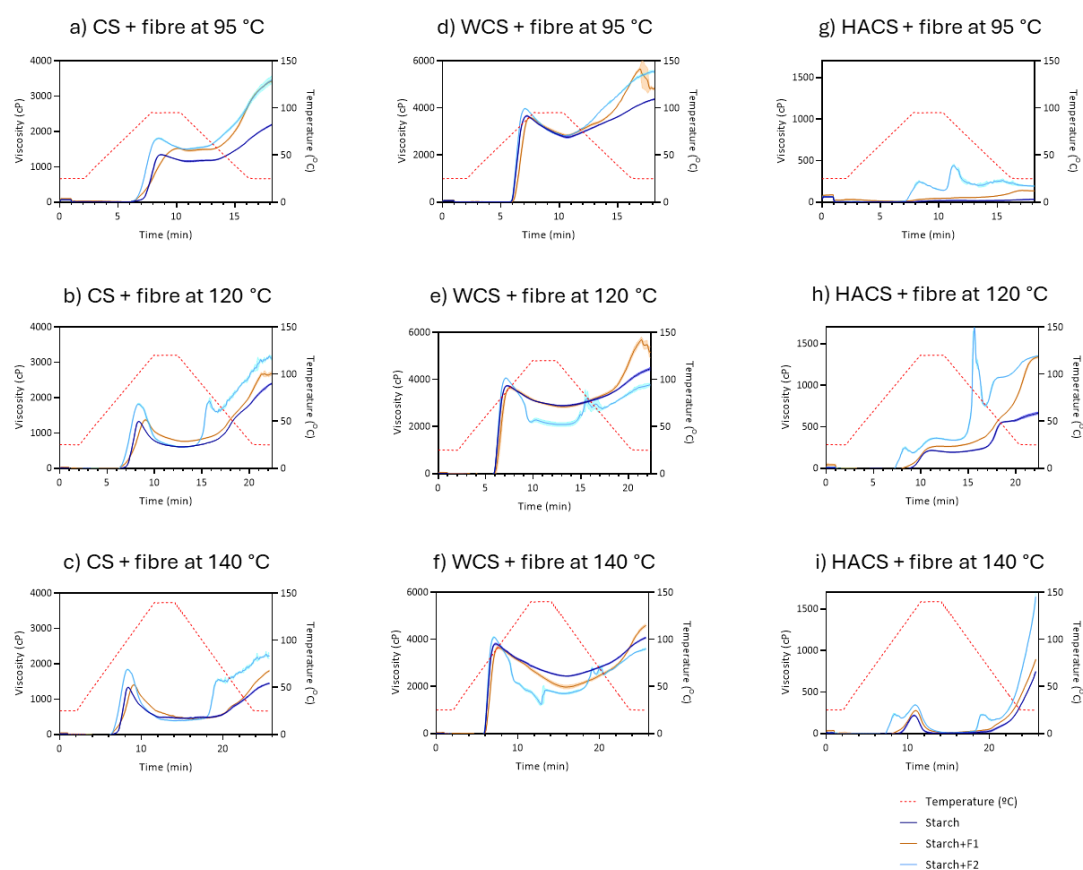


Figure 2. RVA profiles of starches and starch-fibre gels grouped per temperature treatment. Dark blue line represents starch, brown line represents starch+F1, and light blue line represents starch+F2.

Table 2. RVA parameters of starch gels and starch-fibre gels. Different letters correspond to significantly different results at $p < 0.05$.

* Pasting temperature corresponds to first peak in HACS-HX profiles at 120 and 140 °C.

		Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Time of peak viscosity (min)	Pasting temperature (°C)	Temperature at peak viscosity (°C)
95 °C	CS	1346±3 cd	1158±18 c	188±18 g	2190±17 e	1033±25 e	8.67±0.05 c	84.72±0.18 a	94.98±0.03 d
	CS+F1	1529±2 b	1438±11 b	91±12 h	3425±38 a	1987±27 b	10.17±0.18 a	76.97±0.92 b	95±0 d
	CS+F2	1809±17 a	1500±13 a	309±14 f	3460±115 a	1960±105 b	8.48±0.04 de	75.8±0.20 b	94.92±0.03 d
120 °C	CS	1328±6 d	606±4 e	721±7 d	2393±21 d	1786±21 c	8.33±0.03 de	83.6±0.05 a	100.88±0.25 bc
	CS+F1	1377±23 d	764±5 d	608±25 e	2678±57 c	1915±60 bc	9.03±0.02 b	75.98±1.02 b	109.35±0.26 a
	CS+F2	1819±27 a	604±13 e	1215±14 b	3121±62 b	2517±75 a	8.28±0.02 e	75.35±0.56 b	100±0.23 c
140 °C	CS	1333±10 d	457±10 g	876±11 c	1446±13 g	989±3 e	8.37±0.02 d	83.92±0.03 a	101.3±0.20 b
	CS+F1	1404±7 c	516±12 f	888±5 c	1802±21 f	1286±9 d	9.06±0.04 b	75.83±0.85 b	109.77±0.44 a
	CS+F2	1841±21 a	387±14 h	1454±13 a	2224±77 e	1837±78 bc	8.30±0.03 de	75.52±0.18 b	100.52±0.30 c
95 °C	WCS	3662±27 de	2755±36 b	907±29 f	4370±21 d	1615±14 d	7.23±0.01 b	71.35±0.36 abc	87.53±0.13 b
	WCS+F1	3586±31 e	2836±44 ab	750±30 g	5727±120 ab	2891±76 a	7.51±0.04 a	72.07±0.56 a	91.08±0.37 a
	WCS+F2	3972±22 b	2809±11 ab	1163±24 e	5542±57 b	2733±65 ab	7.06±0.03 c	70.52±0.21 bcd	85.63±0.39 c
120 °C	WCS	3736±18 cd	2892±14 a	844±11 fg	4453±65 cd	1561±51 d	7.26±0.03 b	70.37±0.26 bcd	87.97±0.32 b
	WCS+F1	3657±57 de	2841±36 ab	816±28 fg	5746±45 a	2904±10 a	7.55±0.11 a	71.42±1.09 ab	91.53±1.28 a
	WCS+F2	4062±17 ab	2086±85 de	1976±87 b	3768±83 f	1682±167 d	7.13±0.02 bc	70±0.05 cd	86.47±0.21 bc
140 °C	WCS	3809±39 c	2437±13 c	1372±26 d	4069±30 e	1632±19 d	7.26±0.012 b	70.43±0.28 bcd	88±0.13 b
	WCS+F1	3652±67 de	1978±81 de	1673±40 c	4590±62 c	2611±27 b	7.57±0.07 a	71.52±0.36 ab	91.83±0.81 a
	WCS+F2	4090±36 a	1697±42 e	2393±36 a	35963±42 f	1900±26 c	7.09±0.03 c	69.85±0.35 d	85.92±0.33 c
120 °C	HACS	216±2 d	23±4 c	23±4 e	663±14 e	469±15 e	11.13±0.10 b	115.1±0.07 a	120.03±0.06 d
	HACS+F1	252±6 c	263±6 b	/	1341±14 b	1078±17 b	10.99±0.012 bc	115.12±0.13 a	120.1±0 d
	HACS+F2	246±13*/ 355±11a	333±10 a	23±2 d	1357±13 b	1105±19 b	11.61±0.11 a	88.18**±0.40 b	120.05±0 d
140 °C	HACS	227±8 d	/	218±1 c	739±9 d	735±16 d	10.73±0.01 d	115.08±0.03 A	129.73±0.12 c
	HACS+F1	276±4 B	12±2 d	264±2 b	890±6 c	878±5 c	10.95±0.04 c	115.25±0.30 A	132.28±0.38 a
	HACS+F2	234±10*/ 345±5a	10±3 e	335±4 a	1645±49 a	1635±52 a	10.887±0.03 cd	88.37**±0.16 b	131.45±0.33 b

As expected, CS, WCS, and HACS show distinctly different pasting curves. CS showed no changes in peak viscosity (1346, 1328, and 1333 cP, at 95, 120, and 140 °C, respectively), but it had a decreasing trend in trough viscosity (1158, 606, and 457 cP at 95, 120, and 140 °C, respectively), which indicates thermal degradation due to temperature increases (Table 2). Trough viscosity is a measure of the lowest viscosity reached during holding stage (when maximum temperature is being held) as the starch granules collapse due to prolonged shearing and heat, therefore reducing viscosity. CS-120 had the highest final viscosity (2393 cP), followed by CS-95 (2190 cP),

and CS-140 (1446 cP) (Table 2). Amorphous starch matrix formed at the end of RVA measurement is dependent on leached starch polymers and swollen granules which retrograde and create starch gel (Balet et al., 2019; Cornejo-Ramírez et al., 2018). With increased temperature, the amount of leached starch polymers also increased. It is possible that at 140 °C, thermal breakdown of leached polymers happened, which could mean that at 120 °C, a more robust network could have formed. At 95 °C, there were fewer polymers leached than at 120 °C. This could explain why CS-120 had the highest final and setback viscosity. Works of Liu et al. (2019) and Tian et al. (2022), show decrease of final viscosity of normal CS with increased temperature (95 to 140 °C). However, our RVA protocol had heating/cooling rate of 12.3 °C/min, while in both of aforementioned works the rate was 6 °C/min, which could have impacted the starch gel properties, as it has been shown that heating/cooling rate affects the starch granule swelling (Balet et al., 2019). There were no changes in pasting temperature of CS as pasting occurred at <95 °C. Temperature at peak viscosity of CS-120 and CS-140 did not differ (100.88 and 101.30 °C, respectively), while CS-95 had lower peak temperature (94.98 °C) (Table 2). This shows that CS had higher viscosity and, therefore, more swelling in high temperature RVA profiles (120 and 140 °C) due to increased polymer leaching.

WCS showed the highest peak viscosity when compared to CS and HACS, as expected (Fig. 2). Waxy starches are known to have higher peak viscosity, more pronounced through viscosity, and lower setback than their conventional counterparts. This is due to the lack of amylose, which slows granule swelling and contributes to setback viscosity by reassociating into a matrix during cooling stage (Hsieh et al., 2019). Moreover, amylopectin (particularly short chained) can easily bind with H₂O molecules via hydrogen bonds during heating, thus causing starch granule swelling and increase in viscosity at lower temperatures than amylose-containing counterparts (Cornejo-Ramírez et al., 2018). WCS showed a slight increase in peak viscosity with increased temperature (WCS-95 = 3662 cP, WCS-120 = 3736 cP, WCS-140 = 3809 cP) (Table 2). At 140 °C, WCS had the lowest trough viscosity (2437 cP, as compared to 2775 cP at 95 °C and 2892 cP at 120 °C) and, consequently, highest breakdown viscosity (1372 cP, as compared to 907 cP at 95 °C and 844 cP at 120 °C). This is

reported in other works as well and is due to disintegration of starch granules and thermal deterioration (Tian et al., 2022). There were no changes in time of peak viscosity, pasting temperature, or temperature at peak viscosity, which suggest that WCS gelatinised below 95 °C. It has been reported that gelatinization temperature of WCS was 71 °C, as analysed by differential scanning calorimetry (Kuang et al., 2017).

Temperature influence is clearly seen in HACS pasting profile (Fig. 2). At 95 °C, HACS did not gelatinise and its RVA profile shows low viscosity. Typical RVA curve with defined peak viscosity can be seen in HACS-120 (216 cP) and HACS-140 (227 cP) (Table 2). This is expected, since high amylose starches have higher gelatinization point, and increased temperature allows gelatinization and granule swelling. This is reported in other works exploring use of high temperature RVA (Liu et al., 2019; Tian et al., 2022). High amylose starches overall have a lower viscosity and swelling properties than their conventional or waxy counterparts due to the lack of amylopectin, which is why at all temperatures, HACS shows lower viscosity than CS or WCS. In a work exploring digestibility of resistant starch gels under high temperature cooking conditions, gel HA50 had comparable amylose content and concentration to ours (amylose - 59.1 and 63.1%; concentration - 7.82 and 7.5%, respectively) showed very similar peak viscosities (240 cP at 120 °C and 251 cP at 140 °C) (Liu et al., 2020). At 140 °C, there was a sharp decrease in trough viscosity, where it reached low viscosity values which could not have been accurately determined. This can be seen in other works as well (Liu et al., 2020; Tian et al., 2022) and can be attributed to thermal degradation. HACS-120 had lower final viscosity than HACS-140 (663 and 739 cP, respectively), which is different from previous findings (Liu et al., 2020). However, our RVA profile ended at 25 °C, which shows starch gel viscosity at room temperatures, while in referenced papers, RVA profile ends at 50 °C, which shows the hot paste viscosity. Our HACS viscosity curve shows that at 50 °C (cooling stage), HACS-120 had 523 cP, while HACS-140 had 98 cP, which agrees with final viscosity results in published literature (Liu et al., 2020). HACS-140 showed higher temperature at peak viscosity than HACS-120 (129.73 and 120.03 °C, respectively), pointing to higher swelling, and therefore viscosity, at higher temperatures.

2) Influence of fibre addition on pasting characteristics of the 3 corn starch gels with varying amylose content

With fibre addition, we see major changes in pasting profiles (Fig. 2). F1 slightly increased viscosity, while F2 had more pronounced viscosity increase and it changed appearance of pasting curves, particularly at 120 and 140 °C. Appearance of a viscosity spike at ~80 °C in the cooling stage can be seen in all starches, but it was the most prominent in HACS, followed by CS, and then WCS (Fig. 2). It is likely that said change is due to strong gelling properties of F2, as mentioned in previous section. Since RVA is conventionally used to measure paste viscosity, rather than gel viscosity, it is possible that the RVA partially breaks the solid gel, and paddle continues to spin without accurately reporting viscosity. In RVA profile, this is seen as turbulent, uneven, and non-smooth curves. In our work, all pasting curves show good reproducibility and mainly smooth lines. Santamaria et al. (2023) have reported that psyllium husk powder addition changed pasting profile of potato and pea starch gels, but not in corn starch. However, they studied gels with higher concentrations of starch than ours (14 vs 7.5 %, respectively), and used whole psyllium, as opposed to fractions used in our work. Another work studying corn starch replacement with whole psyllium showed RVA curve irregularities when the replacement level was 10% (Belorio et al., 2020). Ren et al. (2020) have reported appearance of additional peak at 85 °C when psyllium powder is added to rice flour, which is very similar to peak appearing in our RVA profile. They also noted that said peak increased with increasing concentration of psyllium. It is possible that psyllium-amylose interactions contribute to the peak appearance, however, when observing RVA profile of psyllium fractions without starches, the peak is very present in F2 (Supplementary Fig. 1). As Ren et al. (2020) have also noted, self-association of psyllium at ~80 °C, temporarily binding and reducing the available water, is a likely explanation, which is supported by our results, as well. F1 showed low viscosity at all temperatures (Supplementary Fig. 1). It has been shown that presence of galacturonic acid can increase solubility of *P. ovata* fractions extracted at low temperature (Yu et al., 2017). To the best of our knowledge, no work was published about incorporation of psyllium fractions to starch gels and their effect on pasting profile.

Fibre addition can increase viscosity of starch during pasting, and this increase is more prominent in starches with conventional amylose content than in waxy starches, which is the case in our work as well. This happens due to synergistic interaction between NSPs and leached amylose (BeMiller, 2011; Zhang et al., 2020). When observing peak viscosity in the 95 °C profile, F1 caused a 14% increase in CS, and no change in WCS, while F2 caused a 34% increase in CS, and an 8% increase in WCS (Fig. 2A and 2D). At high temperature treatments, WCS-F2-120 and WCS-F2-140 had higher peak viscosity than corresponding WCS gels (by 9 and 7%, respectively), but lower trough viscosity (by 28% at 120 °C, and by 30% at 140 °C) (Fig. 2E and 2F). This is most likely due to phase separation – increase in peak viscosity could be due to WCS granules swelling at high temperature which then increases the F2 concentration in continuous phase, leading to higher viscosity. That is followed by the holding phase of the RVA profile in which phase separation and thermal deterioration causes lower viscosity in WCS-F2 than in WCS, even though solids concentration is higher in WCS-F2 than WCS (7.87 and 7.5%, respectively). As presented in previous chapter, we also saw decrease in viscoelastic properties when *P. ovata* mucilage fraction was added to waxy corn starch, which we described as antagonistic interaction. In HACS, there are clear differences when applying the two fractions - F1 increased overall viscosity, while it seems that F2 viscosity behaviour overwhelmed the pasting properties of HACS, especially at 120 and 140 °C (Fig. 2H and 2I). When F2 is added to HACS, a peak appears before the peak viscosity (minute 9, Fig. 2H and 2J). In HACS-F2-120 viscosity of said peak is 246 cP, while in HACS-F2-140 it is 234 cP (Table 2). This is, again, probably a result of F2 having stronger viscosifying effects than HACS, as this early peak is visible in RVA profile of F2 by itself (Supplementary Fig. 1). When soluble and insoluble fibre from corn bran was added to CS, contrasting trends were reported – soluble fibre decreased overall viscosity, while insoluble fibre caused an increase (S. Li et al., 2022). Even though corn bran fibre is very different to *P. ovata* fibre, as it contains cellulose, hemicellulose, lignin, and xylooligosaccharides, this work demonstrates that differently extracted fibre from the same source can cause varied results when added to starch gels. The soluble fibre inhibited starch gelatinisation and decreased the gel strength which contributed to the stability of starch gels. The authors described hydrogen bonding between the starch and soluble fibre, while

insoluble fibre was embedded into the starch network (Li et al., 2022). On the other hand, when one type of fibre is added to different starches, the effect is dependent on specific starch-fibre combinations and probably their interactions. For example, incorporation of xanthan gum to CS, WCS, or HACS caused viscosity increase due to different mechanisms of interaction, as is seen in our work as well (Hong et al., 2015). In xanthan-CS, interaction between xanthan gum and amylose led to increase in peak viscosity, while in HACS gel xanthan caused viscosity increase because HACS could not be pasted by RVA at 95 °C. In xanthan-WCS, phase separation caused higher viscosity, with xanthan gum being highly concentrated in continuous phase as starch granules swell during heating. Similar conclusions were made by Zhang et al. (2020), who studied two types of HACS as well as WCS in combination with xanthan gum and guar gum treated at 130 °C. They suggested that phase separation was a likely driver of higher viscosity when gums were added to WCS. Guar gum showed synergistic effects with HACS with 50% amylose, while HACS with 70% amylose was less affected by gum addition, suggesting that amylose had had a dominant effect on the pasting behaviour (Zhang et al., 2020). Setback viscosity was increased when F1 and F2 were added in all starches at all treatments (Table 2). This could indicate faster amylose retrogradation when fibre is added. Higher setback viscosity are also reported when corn bran arabinoxylans are added to CS, and this is explained by higher local density of leached amylose caused by fibre network which advances amylose nucleation and crystal growth rate (Zhang et al., 2023).

Pasting temperature (PT) is the temperature at which viscosity starts to increase (due to granule swelling and amylose leaching) and starch starts to form paste (Balet et al., 2019). F1 and F2 decreased PT of CS at all temperatures (by approximately 9 °C for both fibres). This is expected, as fibre addition increases viscosity and, thus, pasting will occur at lower temperature. However, F1 and F2 had contrasting effect on PT in WCS – F1 showed slight increase, while F2 showed a slight decrease. WCS-F1 and WCS-F2 were different from each other, but not from WCS at corresponding temperatures. Moreover, changes in WCS were less pronounced (by 1-2 °C), again indicating that amylose-fibre interactions drive pasting behaviour in CS. It has been proposed that amylose-fibre synergistic interaction are greater determinants of

viscosity increase than phase separation (Zhang et al., 2020). In HACS, F1 did not change PT. Due to the aforementioned peak appearance (minute 9, Fig. 2H and 2J) in HACS-F2 gels, PT is recorded as significantly lower (88 °C in HACS-F2, 115 °C in HACS), however this may not reflect actual changes in PT of the starch itself. Altogether, changes in PT are dependent on starch-fibre combination. Addition of F1 delayed peak viscosity and the largest difference can be seen when F1 is added to CS at 95 °C: time of peak viscosity of CS was 8.67 min, while CS-F1 was 10.17 min. Contrarily, F2 addition either did not have an effect or it caused slightly earlier peak viscosity time. Same trend was reported for WCS (F1 delayed peak viscosity, while F2 accelerated it), but the difference was 20-30 seconds (Table 2). In HACS, influence of fibre of time of peak viscosity did not show any trend.

Overall, RVA pasting profiles show that increased temperature treatments affected each starch differently. Fibre addition mostly led to higher viscosity, albeit there are clear differences between F1 and F2 effect on individual starch, and between starch-fibre combinations.

3.3. Techno-functional properties: texture analysis and freeze-thaw syneresis

Fig. 3 shows texture profile (hardness and adhesiveness) of the starch and starch-fibre gels, except HACS at 95 °C as it did not form a gel. WCS did not form a self-standing gel as well, therefore its hardness could not be adequately measured and is not reported. It is observable that fibre mostly did not have a significant impact on texture properties, while temperature affected HACS, CS (hardness), and mostly did not affect WCS. Increase in temperature negatively affected HACS, and gels treated at 140 °C showed significantly higher hardness.

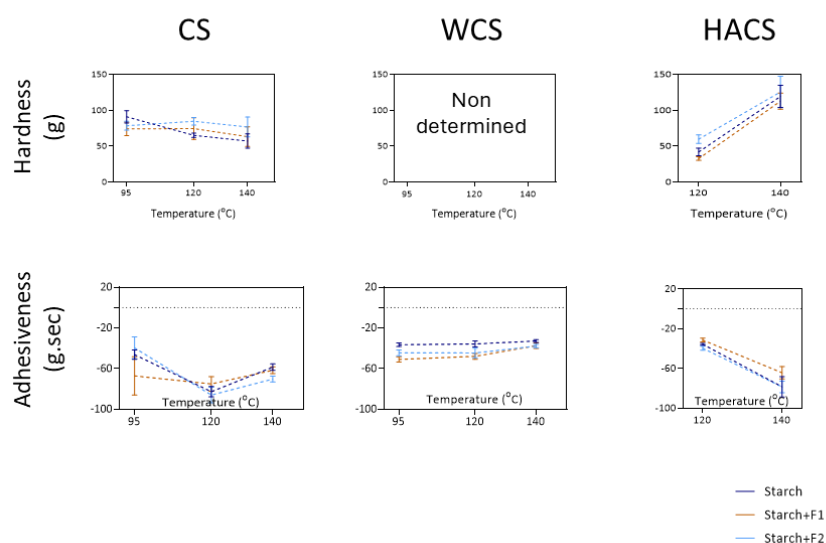


Figure 3. Texture analysis parameters (hardness and adhesiveness) of all gels at 3 temperature treatments. Dark blue colour represents starch gels, brown colour represents starch+F1, while light blue represents starch+F2 gel. Lines are presented to guide the eye.

Tian et al. (2022) employed the use of high temperature (from 95 °C to 140 °C) RVA on a range of starches, and found that higher temperature treatment increased hardness in two high amylose maize starches, while other types of starches were not affected. This agrees with our results. Similar trends can be seen in Liu et al.'s work (2019) – hardness of two high amylose starches (HA50 and HA70) increased as the temperature increased. High amylose starches are gelatinised by high temperature, which leads to granule swelling and amylose dispersion. Upon cooling amylose re-associates into denser, stronger matrix. Therefore, hardness of HACS at 140 °C is higher than at 120 °C (in our work the increase is 184.2%) (Supplementary Table 1). There is a positive correlation between hardness and leached amylose, and correlation coefficient was 0.85 (leached amylose results are discussed in section 3.5.) (Supplementary Fig. 2C). This confirms the proposed theory that increased leached amylose and granule swelling and breakdown results in gels with increased hardness. Adhesiveness of waxy corn starch slightly decreased in Liu et al.' results (2019), whereas our findings showed that adhesiveness of WCS was not affected by temperature. There was a negative correlation between setback viscosity and adhesiveness (-0.74) (Supplementary Fig. 2B). Considering that increases in setback

viscosity generally describe the tendency of amylose and amylopectin to re-align and reassociate into more ordered structure during cooling (Balet et al., 2019), this observation is not surprising. Liu et al.'s (2019) results showed that hardness in normal maize starch significantly decreased, which they contributed to thixotropic breakdown and thermal degradation at 140 °C and this relates to our findings as well (hardness of CS was 91.1 g at 95 °C, 65.1 g at 120 °C, and 57.1 g at 140 °C) (Supplementary Table 1).

Effect of F1 and F2 on starch gel texture mostly did not differ from each other. Noticeable differences between HACS-F1 and HACS-F2 can be seen at 120 °C, where F1 resulted in lower hardness (by 83.2%). Those differences could be explained by F2 showing higher water absorption and swelling properties, as well as higher final viscosity as measured in RVA, which can contribute to hardness. However, there was no difference between HACS-F1 and HACS-F2 at 140 °C, possibly due to thermal degradation of HACS at higher temperature and decreased viscosity of the fibres at 140 °C (Supplementary fig. 1).

Different levels of corn starch replacement (2, 5, 10%) with psyllium was explored in gels treated at 95 °C (Belorio et al., 2020). 2% replacement increased hardness, while 10% decreased it, however, it is difficult to discern whether the changes are caused by addition of psyllium, decrease in starch quantity, or both. In our work, psyllium was added (5.3% addition based on starch), rather than replaced, and it reduces hardness at 95 °C, but not significantly (F1 decreased hardness by 18.6%, and F2 by 13.7%, (Supplementary Table 1). It is known that products with an addition of psyllium require increased water content in order to maintain appearance and texture (Fratelli et al., 2018), however interaction of psyllium with amylose and amylopectin, and consequent effect on texture, still needs to be explored.

Another important factor in determining quality of starch-based products is freeze-thaw stability as it is an indicator of retrogradation and stability of said products during storage. Results shown in Table 3 demonstrate no significant changes in CS and WCS, irrespective of temperature treatment or fibre addition. F1 has shown slight decreasing trend in syneresis when added to CS, which was proportional to increase in temperature (CS-F1 syneresis was 5.23, 4.14, and 3.69% at 95, 120, and 140 °C,

respectively). However, this decrease was not significantly different from CS at corresponding temperature (Table 3). On the other hand, HACS showed high syneresis after 15 days of freeze-thaw treatment, which is due to its high amylose content. Amylose is the primary driver of retrogradation, as it initiates short-term retrogradation (Krystyjan et al., 2022). Fibres reduced syneresis in HACS gels (Table 3). At 120 °C treatment, HACS showed 11.93% syneresis, and F1 reduced it by 41.24%, At 140 °C, HACS had 13.19% syneresis. HACS-F1-140 had 31.39% lower syneresis, and HACS-F2-140 had 37.07% lower syneresis. It was shown that WCS (amylose – 0.75%) had 10x lower syneresis than two types of CS with conventional amylose content (21.61 and 20.48%) at the end of 90-day cold storage (Krystyjan et al., 2022). Xanthan gum addition (0.02 and 0.05%) showed protective effect on the two CSs, while it increased syneresis when added to WCS. This indicates that xanthan gum could slow down short-term retrogradation by restricting mobility of the amylose molecules (Krystyjan et al., 2022). Psyllium has been shown to significantly improve syneresis when added to food products. For example, when psyllium was added to fruit jellies, it completely reduced syneresis (from 11.69 g/ 100g for control sample, to 0 g/ 100g) (Figuerola & Genovese, 2019). Moreover, when combined with other fibres (apple, bamboo, and wheat) who have shown moderate reduction or even increase in syneresis, psyllium-fibre combination again showed no occurrence of syneresis.

Table 3. Freeze-thaw syneresis of starch and starch-fibre gels at 3 temperature treatments. Different letters correspond to significantly different results at $p < 0.05$.

	95 °C			120 °C			140 °C		
	CS	CS+F1	CS+F2	CS	CS+F1	CS+F2	CS	CS+F1	CS+F2
Syneresis (%)	4.11 ±0.86 ab	5.23 ±0.17 a	4.78 ±0.18 ab	4.14 ±0.12 ab	4.14 ±0.05 ab	3.92 ±0.24 b	4.59 ±0.36 ab	3.69 ±0.21 b	4.75 ±0.83 ab
	WCS	WCS+F1	WCS+F2	WCS	WCS+F1	WCS+F2	WCS	WCS+F1	WCS+F2
Syneresis (%)	4.76 ±0.72 ab	4.39 ±0.49 ab	4.73 ±0.66 ab	3.97 ±0.38 b	4.04 ±0.48 b	4.46 ±0.34 ab	5.63 ±0.62 a	4.43 ±0.57 ab	4.29 ±0.31 ab
	HACS	HACS+F1	HACS+F2	HACS	HACS+F1	HACS+F2	HACS	HACS+F1	HACS+F2
Syneresis (%)	/	/	/	11.93 ±0.68 ab	7.01 ±0.48 c	11.81 ±2.38 ab	13.19 ±1.06 a	9.05 ±0.34 bc	8.30 ±0.12 c

Overall, both fractions show limited effects on the hardness and adhesiveness of the tested starches. Moreover, CS and WCS were not affected by temperature treatment, unlike HACS. While the presented texture analysis (penetration test) did not show significant changes (Supplementary Table 1), the fibre addition may have advantages in compression texture profile analysis. Freeze-thaw syneresis measurements have shown no changes in CS and WCS, whereas fibres had positive effect (meaning lower water separation) in HACS, which is desirable in food product development.

3.4. SEM

Scanning electron microscopy (SEM) is oftentimes used for assessing starch gel structure. However, this technique has certain limitations. As explained in the work of Ren et al. (2024), starch gels cannot be used in hydrated state, but the microstructure is changed by freezing and drying. Drying causes shrinkage, and we cannot adequately evaluate the distribution of starch and water (and fibre, if included). Visible pores are result of removal of ice crystals, rather than the result of starch structure itself and, lastly, we cannot differentiate between ordered and disordered parts of starch gel (Ren et al., 2024). Nevertheless, SEM can give us indication of differences in microstructure between samples, as the size, shape, and distribution of ice crystals formed during freezing are dependent on structural differences between starch and starch-fibre gels.

SEM images of the gels explored in this work are presented in Fig. 4.

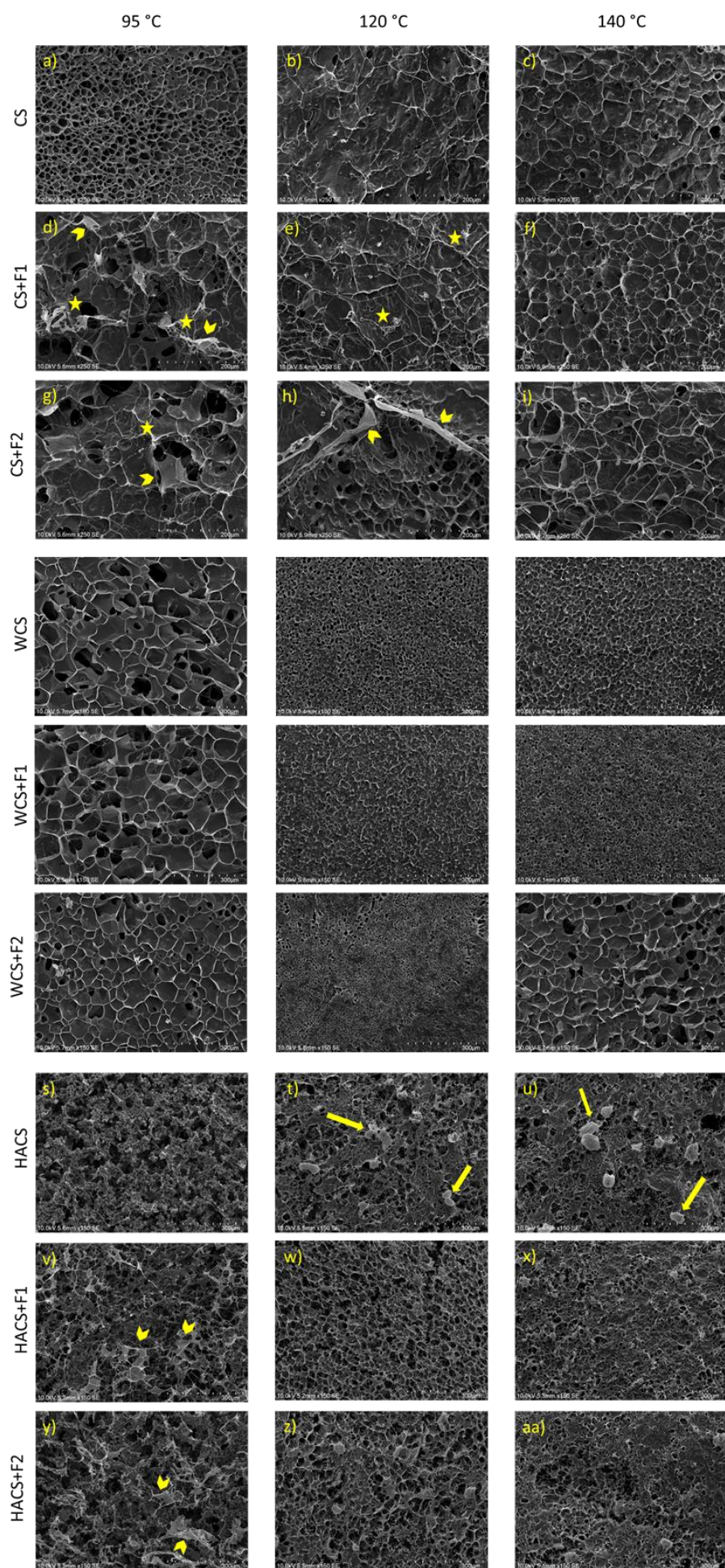


Figure 4. Scanning electron micrographs (SEM) of starch and starch-fibre gels. Temperature treatments specified at the top of the figure, and corresponding samples are written on the left-hand side. Stars point to fibre clusters, chevrons point to sheet-like fibre structures, and arrows point to ungelatinized starch granules.

In CS-95, we can see uniform distribution of pores, whereas in CS-120 and CS-140 the structure appears flakier (Fig. 4A-C). This change in structure agrees with other published literature (Liu et al., 2019). In CS-120 and CS-140 (Fig. 4B and 4C), spider-like filaments are visible, which is probably leached amylose (Ren et al., 2024). In WCS (Fig. 4J-L), higher temperature treatments caused pronounced shrinkage of pores. SEM images show changes in formed ice crystals. WCS-95 shows ice crystals formation in the starch matrix (pore area of $4226 \pm 1275 \mu\text{m}^2$), while in WCS-120 and WCS-140 interpenetration of water molecules in starch matrix suppresses ice crystallization and pores are smaller (319 ± 76 and $456 \pm 86 \mu\text{m}^2$, respectively). At 95 °C, WCS structure (Fig. 4J) seems loosely packed, which is noted in Tian et al.'s work as well (2022). When studying the effect of high temperature RVA on starch gel structure, they described conventional and waxy starches as having loosely packed and flaky structures, and high amylose starches as granular aggregates (Tian et al., 2022). In line with this, for HACS (Fig. 4S-U), it is evident that at 95 °C, it did not gelatinise as starch granules are easily visible. At 120 and 140 °C, the HACS matrix is compact, which indicated gelatinisation, with some swollen starch granules visible (Fig. 4T and 4U, marked with arrows).

The addition of F1 and F2 impacted structure of all three starches in different ways. In CS (Fig. 4D-J), both fibres can be seen as spider-web on the surface of starch, and as entanglements (marked with stars) and sheet-like areas (marked with chevrons) at 95 and 120 °C (Fig. 4D, 4E, 4G, 4H). At 140 °C (Fig. 4F and 4J), both fibres show similar web-like matrix, which points that the higher temperature combined with shear (160 rpm) might have dispersed the fibre, thus forming more uniform web fibre structure. In WCS (Fig. 4M-R), the fibre 'web' is less visible, which is possibly due to amylopectin network of the starch predominating fibre network. Interestingly, in WCS-F2-120 (Fig. 4Q), a change in distribution of pores could indicate phase separation as, most likely, there is a change in size of ice crystals formed during freezing and subsequently dried,

leaving pores at the surface. In WCS-F2-140 the pore size is larger than in WCS-F2-120 (1915 ± 752 and $109 \pm 29 \mu\text{m}^2$, respectively). The pores are more similar to WCS-F2-95 ($3237 \pm 1185 \mu\text{m}^2$), however they seem deeper, which could indicate structure deterioration by higher temperature treatment. A similar observation can be seen in Liu et al.'s work (2019), where waxy maize starch heated at 140°C had deeper, larger, and more elongated pores. In HACS, sheet-like fibre structures can be seen for both F1 and F2 at 95°C (Fig. 4V and 4Y, marked with chevrons), and at higher temperature treatments, a dense fibre matrix is apparent (Fig. 4W, 4X, 4Z, 4AA). It seems that addition of F1 could have led to increased gelatinization since there are less ungelatinized starch granules, whereas in HACS-F2 at 120 and 140°C more ungelatinized starch granules are visible. When whole psyllium husk was added to wheat starch and treated at 90°C for 10 min, it showed an irregular and rough surface (Atac & Sensoy, 2022). The surface appears different from what we observed, however there were clear differences in starch-fibre gels: use of whole vs fractionated psyllium, ratio of psyllium to starch (1:1 in referenced paper), and sample preparation. As for SEM analysis of starch-fibre gels treated at high temperatures, Zhang et al. (2020) have studied the addition of guar gum and xanthan gum in two types of HACS and in WCS heated up to 130°C . Their SEM images show that guar gum may promote amylose aggregation, and xanthan gum forms denser structure in HACS, but neither of added gums had significantly changed WCS structure. Our images show different trend, where both F1 and F2 did change WCS structure at 140°C . However, psyllium (and the fractions used in this work) is different from guar gum and xanthan gum, so difference in WCS-fibre structure is not surprising.

3.5. Leached amylose

Leached amylose can be defined as amount of amylose that leaches from swollen starch granules into surrounding continuous phase during heating (above gelatinization) and shearing of starch paste (Doblado-Maldonado et al., 2017). Fig. 5 shows leached amylose (%) of CS and HACS relative to total starch (Fig. 5A) and relative to the amylose content of the individual starch (Fig. 5B). There was no detected leached amylose for WCS (not shown). The increase in temperature increased leached amylose in both starches, while fibre addition had different effects.

In CS, addition of F1 significantly decreased amylose leaching only at 95 °C (by 22.56%), and F2 significantly decreased amylose leaching at 95 °C and 120 °C (by 18.19% and 10.99%, respectively). In HACS, the addition of F1 significantly increased leached amylose at 120 °C and 140 °C (by 65.44% and 8.30%, respectively), and HACS-F2 was not different from HACS at any temperature treatment. At 95 °C, there was no significant difference, which can be attributed to HACS not reaching gelatinization at 95 °C. It is also evident that majority of amylose content of CS leached out of starch granules at all temperatures (73.66% and 87.67% of amylose was leached at 95 °C and 140 °C, respectively), while for HACS only at 140 °C resulted in relatively high amylose leaching (57.12%).

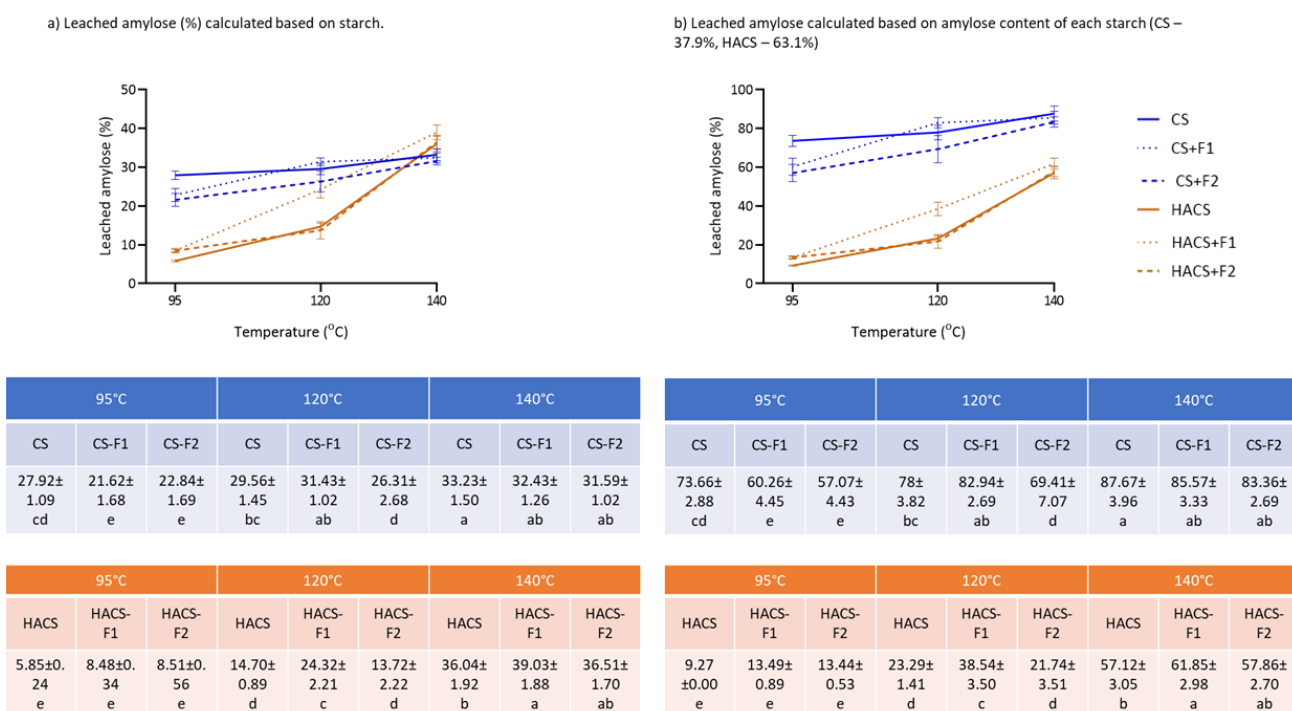


Figure 5. Amylose leaching in CS with and without fibre addition, and amylose leaching in HACS with and without fibre addition calculated: A) based on starch content, B) based on amylose content of individual starch (CS – 37.9%, HACS – 63.1%). Lines are drawn to guide the eye. There was no leached amylose detected in WCS. Different letters correspond to significantly different results at $p < 0.05$.

Effects of fibre or gums on amylose leaching in variety of starches have been reported. However, a need for higher temperature treatment of HACS for gelatinization and

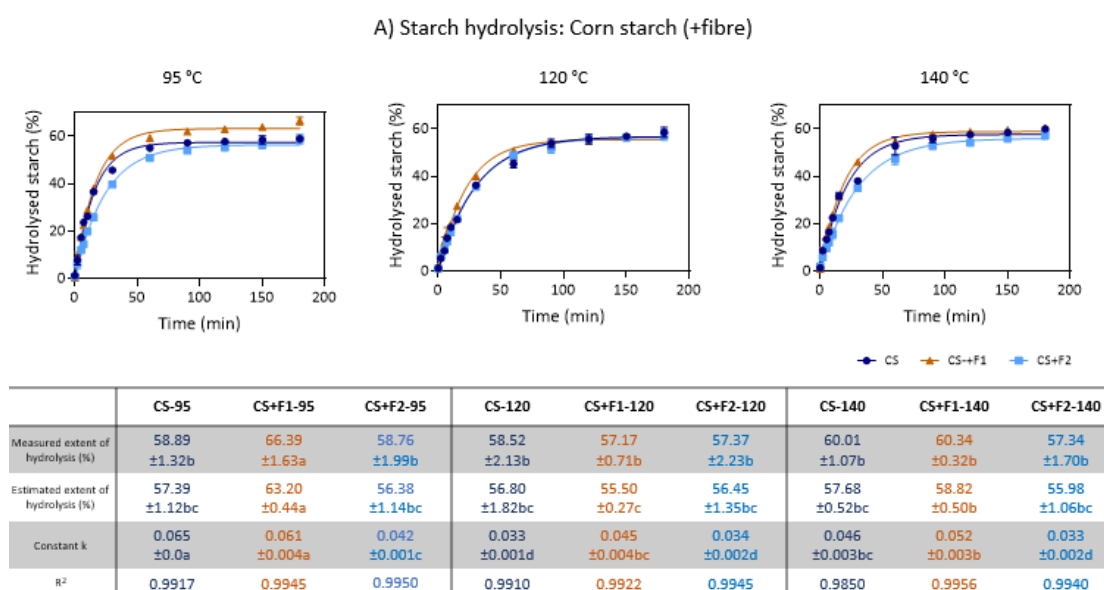
insufficiency of conventional gelatinization methods (such as RVA at 95 °C or hydrothermal treatment in water bath or on stirrer plate under 100 °C) has resulted in a lack of available literature on amylose leaching of HACS. Temperature treatment and heating rate have a pronounced effect on the amount of leached amylose (Doblado-Maldonado et al., 2017). Considering that rate of heating was kept constant in our experiments (Section 2.3.), it can be concluded that increased holding temperature has resulted in increased leached amylose in both CS and HACS, which agrees with literature (Doblado-Maldonado et al., 2017). When wheat AXs (0.5%) of different Mw were added to wheat starch, it was shown that lower Mw (up to 373 kDa) did not affect amylose leaching, but wheat AX with higher Mw (482, 421, and 754 kDa) decreased amylose leaching (Yan et al., 2022), which the authors attributed to the restriction of gelatinization by higher Mw AXs as they could decrease the amount of available water. Similarly, corn fibre gum (0.5%) of varying Mw (186, 290, and 338 kDa) reduced amylose leaching in wheat and corn starch, with greater effect seen in corn starch (Qiu et al., 2016). Adsorption of corn fibre gum onto the starch granule surface and a possible interaction with amylose were proposed as explanations. Both wheat AX and corn fibre gum could be compared with F2 in our work – compositionally, they are arabinoxylans (with a minor galactose component), although they do have different A:X ratios (F2 had lower than wheat AX and corn fibre gum) and similar mechanisms could be at play in our CS-F2 system.

As mentioned, HACS-F1 has shown a contrasting effect on amylose leaching – F1 significantly *increased* the amount of leached amylose at 120 and 140 °C, with the most apparent increase at 120 °C (60.44% increase from HACS at 120 °C). Thus, different mechanisms are at play than the ones between HACS and F2 (which did not show significant difference in amylose leaching). Monosaccharide composition (Fig. 1C) and other published literature (Ren, Yakubov, et al., 2020; Yu et al., 2017) has shown presence of pectin (9.6%) and low branched heteroxylan (A:X = 0.17). Densely branched wheat AX (A:X ranging from 0.79 to 0.97) has been shown to have a lowering effect on amylose leaching in wheat starch (Xie et al., 2022). Pectin has been shown to decrease amylose leaching in wheat, potato, and corn starch (Chen et al., 2023; Yin et al., 2021; Zhang et al., 2021). Therefore, it is likely that low branched HX

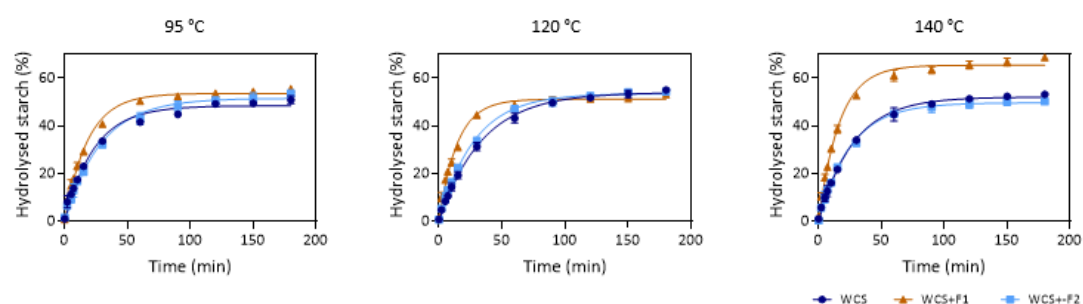
in F1 has a more important role in amylose leaching increases than the presence of pectin in F1.

3.6. Starch hydrolysis by α -amylase and estimated glycaemic index (eGI)

Apart from the techno-functional alterations to starch gels by the addition of fibre, it is also possible that the digestibility of the starch product may be impacted and thus effect the nutritional properties. Starch hydrolysis by α -amylase is a useful predictor of metabolic response (Edwards et al., 2019), as hydrolysis rate (constant k) and extent of hydrolysis (C_{∞}) of a starch are clearly affected by fibre addition and temperature. Psyllium husk is known as a modulator of glycaemic response and has positive effect on glycaemic control, specifically in diabetes mellitus 2 patients (Gibb et al., 2015). However, studies of psyllium and its fractions in *in vitro* enzymatic starch hydrolysis are sparse. Here we present, to our knowledge, first results of enzymatic hydrolysis of corn starch with varying amylose and added psyllium fractions. In Fig. 6, we show non-linear fitting model of hydrolysed starch and starch-fibre gels, as well as associated hydrolysis parameters.

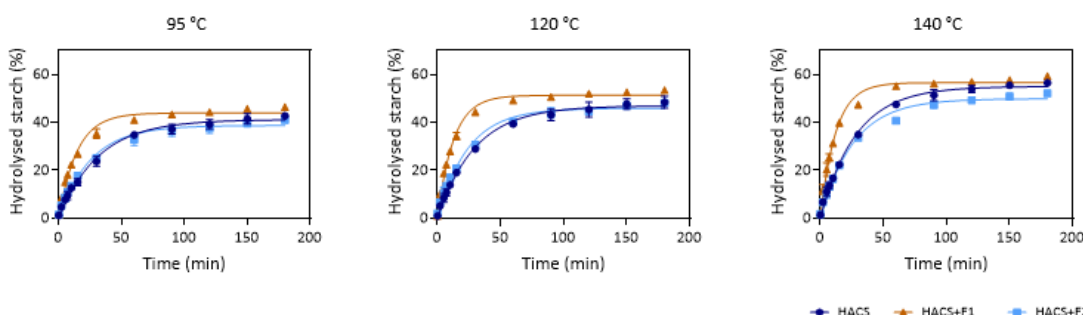


B) Starch hydrolysis: Waxy corn starch (+fibre)



	WCS-95	WCS+F1-95	WCS+F2-95	WCS-120	WCS+F1-120	WCS+F2-120	WCS-140	WCS+F1-140	WCS+F2-140
Measured extent of hydrolysis (%)	50.81 ±1.63cd	55.50 ±0.59b	53.58 ±1.00b	55.00 ±0.69b	53.14 ±0.87bc	54.26 ±0.46b	53.14 ±0.94bc	68.60 ±0.67a	50.12 ±1.06d
Estimated extent of hydrolysis (%)	48.38 ±1.08e	53.56 ±0.29bc	51.54 ±1.24bcd	53.97 ±0.45b	51.14 ±0.80cd	53.49 ±0.65bc	52.09 ±0.70bcd	65.40 ±1.47a	49.63 ±1.10de
Constant k	0.043 ±0.004c	0.055 ±0.005b	0.035 ±0.003cd	0.029 ±0.002d	0.070 ±0.004a	0.036 ±0.003cd	0.036 ±0.004cd	0.060 ±0.005ab	0.040 ±0.003cd
R ²	0.9826	0.9902	0.9871	0.9954	0.9875	0.9953	0.9937	0.9910	0.9937

C) Starch hydrolysis: High amylose corn starch (+fibre)



	HACS-95	HACS+F1-95	HACS+F2-95	HACS-120	HACS+F1-120	HACS+F2-120	HACS-140	HACS+F1-140	HACS+F2-140
Measured extent of hydrolysis (%)	42.76 ±0.94ef	46.32 ±0.82f	40.99 ±1.28de	48.38 ±2.52d	53.39 ±0.62bc	48.10 ±1.29d	56.44 ±0.97ab	59.22 ±1.14a	52.05 ±0.58c
Estimated extent of hydrolysis (%)	41.09 ±1.27ef	43.90 ±0.75de	38.69 ±1.60f	46.85 ±2.34cd	51.26 ±0.60b	45.85 ±1.03d	54.96 ±0.90a	56.51 ±0.19a	49.83 ±0.07bc
Constant k	0.032 ±0.003c	0.069 ±0.006b	0.039 ±0.003c	0.033c	0.079 ±0.005ab	0.042 ±0.003c	0.035 ±0.002c	0.082 ±0.009a	0.038 ±0.004c
R ²	0.9850	0.9807	0.9791	0.9884	0.9898	0.9836	0.9921	0.9855	0.9863

Figure 6. Starch hydrolysis by α -amylase of starch and starch-fibre gels (CS – a), WCS – b), HACS – c)) with corresponding temperature treatments written on top of the figure panels. Dark blue line represents starch, brown line represents starch+F1, and light blue line represents starch+F2. Results shown are: non-linear fitting of starch hydrolysis curve, and starch hydrolysis parameters (measured and estimated extent of hydrolysis, hydrolysis rate, and R²) in corresponding tables below. Different letters correspond to significantly different results at p<0.05.

When observing CS gels (Fig. 6A), temperature treatment did not change the hydrolysis extent, while constant k was highest at 95 °C treatment, followed by 140 °C, and lastly 120 °C (0.065, 0.046, 0.033, respectively). The addition of F1 increased hydrolysis extent at 95 °C (by 12.7%), and it increased hydrolysis rate at 120 °C (by 36%). There were no significant changes between CS and CS-F1 starch hydrolysis at other conditions. As for the addition of F2, decreases in constant k were found in 95 and 140 °C treated samples (by 35.4 and 28.3%, respectively), though F2 did not change the extent of hydrolysis at any temperature. Overall, the most similar starch hydrolysis profiles between CS and CS with fibres can be noticed at $T_{\max}=120$ °C.

Hydrolysis extent in WCS (Fig. 6B) was the lowest at 95 °C (48.38%), and it did not differ significantly between 120 and 140 °C treatments (53.97 and 52.09%, respectively). The lowest constant k was measured in 120 °C treatment (0.029). The addition of F1 significantly increased constant k at all temperatures, with the highest increase at 120 °C (141.4% increase). The highest change in hydrolysis extent can be seen at 140 °C, where addition of F1 resulted in a 29.1% increase. F2 did not significantly change constant k , and it had an inconsistent effect on hydrolysis extent (increased at 95 °C by 9.2%, and no change at 120 and 140 °C).

Starches with high amylose content require higher temperatures to achieve gelatinization (H. Li et al., 2019), therefore utilising HT-RVA gives us useful insight into hydrolysis behaviour of HACS with and without fibre. As expected, with increased temperature, both rate and extent of hydrolysis increased, in gels with and without fibre (Fig. 6C). F2 did not significantly change hydrolysis rate of HACS at any condition, and it decreased hydrolysis extent only at 140 °C (by 7.8%). Contrarily, F1 showed high hydrolysis rate increase (compared to HACS only, F1 increased constant k by 115.6%, 139.4%, and 134.3% at 95, 120, and 140 °C, respectively). Moreover, F1 caused increase (by 8.3%) in hydrolysis extent at 120 °C, while it had no effect at 95 and 140 °C.

Generally, F1 changed starch hydrolysis patterns by increasing hydrolysis rate in almost all cases (except CS-95 and CS-F1-95, where there was no difference), with the biggest changes in HACS gels. F2 had inconsistent effects: it mostly did not significantly change hydrolysis rate, except in CS-F2-95 and CS-F2-140, where it

showed decrease compared to CS gels. As for hydrolysis extent, addition of F1 either increased or did not change hydrolysis extent, and F2 either decreased or did not have an effect.

As one of the most globally consumed crops, corn digestibility is aimed to be modified (Bello-Pérez et al., 2021) with the goal of reducing glycaemic index (GI). In literature, we can find reports of additions of different NSP to corn starch. Some researchers report methodology similar to ours with results showing k and C_{∞} , while others utilise Englyst assay (Englyst et al., 1992) which reports rapidly digested starch (RDS), slowly digestible (SDS) and resistant starch (RS). Even though we cannot directly compare those results to ours, we can cautiously juxtapose trends in RDS and RS with trends in hydrolysis rate and extent - starches with high hydrolysis rate and RDS can lead to spike in blood glucose level, and starches with high RS and low hydrolysis rate and extent can improve glycaemic control (Xie et al., 2018).

At 95 °C treatment CS showed the highest hydrolysis extent and rate, followed by WCS, then HACS. This agrees with work published by Zheng et al. (2021) who assessed *in vitro* sequential dynamic digestion of CS, WCS, and HACS gels treated at 95 °C for 30 min. In the intestinal stage, hydrolysis of gastric digesta (180 min) of the three starches followed the same trends as in our results (CS>WCS>HACS). They explained that HACS gel contained native starch granules which are less susceptible to enzymatic attack, while highly viscous WCS was more difficult to hydrolyse by α -amylase.

Liu et al. (2020) reported that resistant maize starch (59.1% amylose) treated using HT-RVA at 120 °C showed highest RDS, and lowest SDS, when compared to treatments at 95 and 140 °C (which did not differ from each other). Their explanation was that at 120 °C, starch granules were not dispersed enough (as shown by SEM) to develop enzymatically resistant structures during cooling (0.5 h). That said, our results do not agree with theirs, as we have shown an expected increase in hydrolysis rate and extent with the temperature increase. To the best of our knowledge, this is the only work that has reported starch digestibility kinetics (with hydrolysis rate and extent) when using high temperature RVA.

Psyllium husk is proven to be beneficial in glycaemic control. When added to wheat, potato, and tapioca starch, psyllium lowered RDS and increased SDS and RS in all three starches (Sevilmis & Sensoy, 2022). Increase in RS was the highest for wheat starch (121 – 132%, depending on water addition). This can be attributed to increased viscosity caused by psyllium and by reducing the amount of water available for gelatinization. However, psyllium addition was 50%, which is quite high and is not feasible in food product formulation. Psyllium showed decrease in starch digestibility of fibre enriched white bread: when compared to xanthan gum and lambda-carrageenan, 3% psyllium addition showed the lowest GI (61, 54, and 46, respectively) (Yassin et al., 2022). Santamaria et al. (2023) have developed a sequential RVA protocol followed by the addition of α -amylase where they studied the relationship between changes in viscosity caused by enzymatic hydrolysis of starch. They reported an increase in hydrolysis rate k when psyllium was added to CS. CS has hydrolysis rate of 0.311 min^{-1} , and when 0.5% and 2.5% psyllium were added, k increased to 0.484 and 0.595 min^{-1} , respectively (Santamaria et al., 2023). It is clear that there is research interest in the effect of psyllium on starch digestibility, but there is less information about how psyllium fractions influence patterns of starch hydrolysis.

There are many works focused the on impact of fibre addition on starch hydrolysis, with most of them reporting positive effect, i.e. reduction of starch digestibility (Ma et al., 2024). When different concentrations of pectin were added to corn starch (0.5 - 10%), decrease in RDS and increase in SDS and RS were proportional to pectin addition (Ma et al., 2019). Corn bran arabinoxylans of varying crosslinking degree decreased hydrolysis rate and extent of corn starch, and highly crosslinked arabinoxylan (H-CLAX) caused lowest k and C_{∞} (1.99×10^{-2} , and 89.76%) (Zhang et al., 2023). This was explained by a high final viscosity which could have inhibited the diffusion of α -amylase. However, changes in hydrolysis rate and extent are often dependent on the physico-chemical properties of fibre or gum which is added to the starch gel (such as molecular weight, branching, charge, and concentration used in gel preparation). On the other hand, some papers report increases in starch hydrolysis susceptibility, which is apparent in our work when F1 was added. For example, when pectin, guar gum, CMC, xanthan, and HPMC were added to corn starch in

concentration of 1-4%, only HPMC (3 and 4%) decreased constant k , while there was a significant increase in all other gels (Gularte & Rosell, 2011). As for the extent of hydrolysis, the effect of gums was not dependent on type or concentration of the added gum. The authors explained that the interaction of amylose and a gum added could prevent retrogradation and therefore facilitate enzymatic attack, but also surmised that increased viscosity caused by gum addition could slow down α -amylase. Therefore, it seems wise to look at each gum-starch combination, as general trends were not found (Gularte & Rosell, 2011). In another work, addition of xanthan and konjac gum (0.05 - 0.3%) to mung bean resistant starch led to increased RDS and SDS, and decreased RS (Lin et al., 2021). Both fibres promoted enzymatic hydrolysis of mung bean resistant starch, and significantly lowered RS fraction which could indicate different digestion patterns when gums are added to resistant starches (as opposed to conventional starches). This concept should be further explored.

In our work, it is clear that different mechanisms play key roles in influencing starch hydrolysis depending on the type of starch and the type of fibre used. Moreover, significant increase of the hydrolysis rate by F1 addition should be additionally reviewed. Here we discuss possible mechanisms.

As shown in Supplementary Fig. 1, F1 had very low viscosity at all 3 temperature treatments. Moreover, addition of F1 did not have a pronounced viscosity increasing effect as F2 did. In the case of WCS, F1 decreased peak viscosity. This points to a possibility of low-viscosity areas in gel structure which could allow for increased relative concentration of α -amylase and, subsequently, its ability to hydrolyse starch faster. Hydrated state of F1 would also have lower volume than F2 (as WSC of F1 is 5.27, and WSC of F2 is 9.67 ml/g), which could lead to more interstitial water, resulting in increased enzyme distribution in areas of interstitial water (as F2 would use more water and form highly viscous phase). This concept has been proposed in works exploring pectin addition to starch (Bai et al., 2021), however this hypothesis has not been proven. More precisely, this possibility could be true for some fibres with specific intrinsic properties, rather than one type of fibre, or only for certain concentrations. This “water distribution” theory might explain results seen in WCS starch hydrolysis. When observing SEM Images of WCS, WCS-F1 and WCS-F2 at 140

°C, there is apparent difference in pore size, which is attributed to ice crystals formation. WCS-F1-140 has smaller pore size ($173 \pm 45 \mu\text{m}^2$, whereas WCS-140 has $456 \pm 86 \mu\text{m}^2$, and WCS-F2-140 has $1915 \pm 752 \mu\text{m}^2$ pore size), which could represent interpenetration of water in matrix (interstitial water phase), and suppression of ice crystals formed when storing the gels at -80°C . As mentioned, this phase could lead to increased relative concentration of amylose, which would lead to increased starch hydrolysis rate and extent (as is reported in Fig. 6B).

Another possible explanation concerning why does F1 increase starch hydrolysis is related to amylose leaching. It has been shown that higher temperature leads to increased amylose leaching accompanied by amylopectin leaching as well (Doblado-Maldonado et al., 2017). We report agreeing findings as well (Fig. 5). F1 could have disrupted amylose network, either by (1) dilution with interstitial water which would lead increased amylose leaching, (2) interfering with amylose network and disrupting amylose-amylose interactions, or (3) interacting with amylose itself which could make amylose chains more susceptible to enzymatic attack. This would explain the increased constant k and hydrolysis rate in HACS at all temperatures. This could also explain increased hydrolysis rate and extent in WCS-F1-140 where perhaps even non-measurable amylose (which would have been leached the most at 140°C) could have caused faster enzymatic attack. Additionally, leaching of low molecular weight amylopectin could have also been increased by temperature and F1, which could explain why F1 seemed to increase both starch hydrolysis parameters in WCS. However, to further elaborate and prove this theory, analysis like size exclusion chromatography would be advisable.

Contrarily, F2 does have higher viscosity, which could have slowed down enzymatic mobility. Also, F2 can act as “shield” around starch, thus making it inaccessible to α -amylase (Zhang et al., 2022). F2 has both WRC and WSC higher than F1, which could mean increased capability of starch encapsulation (WRC of F1 is 12.03, and of F2 is 28.42 g/g; WSC of F1 is 5.27, and of F2 is 9.67 ml/g; Fig. 1B). Moreover, due to its high WRC, F2 could limit the amount of water available for starch gelatinisation, thus leading to decreased susceptibility to enzymatic hydrolysis. This could be the case for HACS, as in SEM images we can see ungelatinized starch granules when F2 is added,

and no granules or smaller amount when F1 is added (in HACS-F1-120 and HACS-F1-140, respectively) (Fig. 4W, 4X, 4Z, 4AA). It has been proven that both physico-chemical properties and viscosity has significant effect on starch hydrolysis – when 4 different soluble fibres (xanthan gum, konjac glucomannan, hull-less barley β -glucan, tamarind seed gum) were added to corn starch at the same initial viscosity level, they showed similar results of starch hydrolysis extent, but significant differences in the digestion rate constant (Zhang et al., 2021). However, whether these possible mechanisms can explain the contrasting effects of F1 and F2 seen here is highly speculative and would require further analyses to show not only the interactions between starches and fibre fractions, but also if the fibre fractions have any interaction with α -amylase.

Glycaemic index (GI) is a measurement of carbohydrate-containing foods and their glycaemic response *in vivo*. GI has been correlated with metabolic health and is dependent on the rate and extent of starch hydrolysis (Edwards et al., 2019). Our eGI calculations (estimated GI) (Fig. 7) show that F1 increased eGI of CS-95, WCS-95, WCS-140, and in all HACS gels. F2 decreased eGI in CS-95 and HACS-140. Unsurprisingly, eGI was significantly positively correlated with the hydrolysis extent for all 3 starches (CS – 0.91, WCS – 0.96, HACS – 0.97), and with hydrolysis rate for CS (0.89) and HACS (0.68) (Supplementary Fig. 2). For WCS, correlation between hydrolysis rate and eGI was positive, but not significant (0.62) (Supplementary Fig. 2B). Considering that eGI is an indicator of glycaemic response, F1 could lead to its increase, which would not be advisable for people who have indication of metabolic syndrome, but might be applicable for developing rapid-energy-supply products for athletes and during high intensity activity, or supplements for malnutrition which, besides rapidly digested starch would have potentially prebiotic fibre addition (Harris et al., 2023). However, use of F2 would be advisable when developing further starch-based products for people with metabolic disorders or in products that are known to have high GI, such as gluten-free bread (Romão et al., 2021).

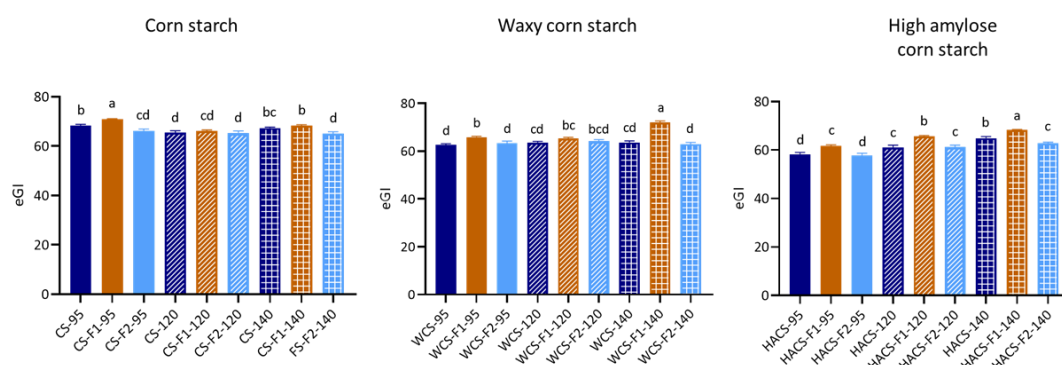


Figure 7. Estimated glycaemic index (eGI) of starch and starch-fibre gels. Dark blue colour represents each starch gel, brown colour represents starch+F2. and light blue represents starch+F2. Full colour columns are treatments at 95 °C, lined columns are treatments at 120 °C, and columns with squared pattern are treatments at 140 °C. Different letters correspond to significantly different results at $p < 0.05$.

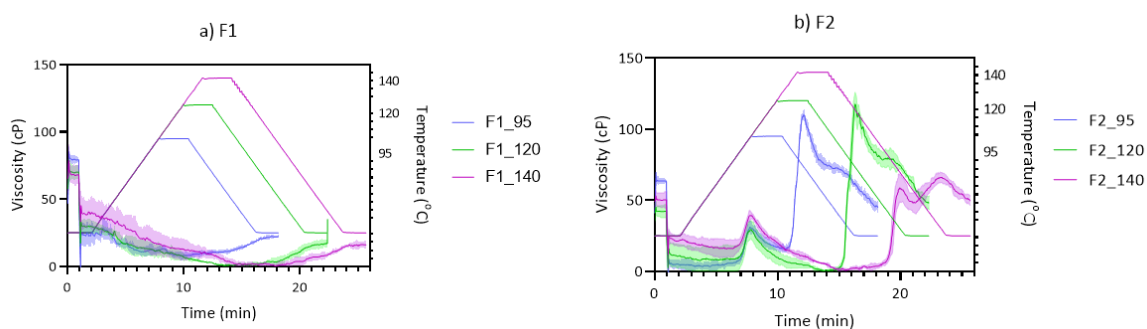
4. Conclusion

To date, no one has studied interaction between starch type, temperature treatment and fibre fractions of *P. ovata* husk. So far, most of the studies have focused on the effect of high temperature on different starches, while the combined effect of fibre and temperature is underexplored. In light of the ongoing efforts to enhance dietary fibre intake and to nutritionally optimize starch-based products, we propose that our research will address the existing knowledge gap regarding the properties and behaviour of starch-fibre gels subjected to high-temperature treatments. This work explored the addition of two fibre fractions (F1 and F2) from *P. ovata* husk in 3 starches with varying amylose content at three different temperatures (95, 120, and 140 °C). The two fractions were heteroxylans with varying pectin content and arabinose to xylose ratio (F1 had 9.6% pectin and A:X=0.17, while F2 had no pectin and had A:X=0.36). F2 was more viscous, and had higher hydrating capacities. F1 and F2 had contrasting effect on starches, which also depended on the starch type, and changed the functionality of starch-fibre gels. F1 slightly increased viscosity of the starches, while F2 changed the appearance of the RVA curve causing several new peaks. Higher temperature caused thermal degradation in all starch types. Both fibre and temperature had limited effects on texture properties and freeze-thaw syneresis.

F1 significantly increased starch hydrolysis rate, which was explained by increase in leached amylose that could have been hydrolysed faster, while F2 had no effect, or it slightly decreased starch hydrolysis parameters.

This work included wide range of starch and starch-fibre gels with focus on analyses which would be of use in food and health industry. For example, highly viscous fibre (F2) would be used for formulation of products with lower glycaemic index, while soluble fibre with low viscosity (F1) could be used for starch based products where fast glucose release is desirable. In addition, for fabricating fibre-enriched products where the requirement is that texture and viscosity during thermal treatments remain similar to the original product, then F1 would be advisable. However, mechanisms that drive the reported results should be further explored. We propose that detailed rheology studies, cryo-SEM and size exclusion chromatography of leached polymers could provide insights into interactions between starches and fibres and the gels' structure. Moreover, using confocal microscopy with fluorescent labelling could explain more detailed differences in structure.

Finally, novelty and significance of this paper lies in studying *P. ovata* fractions, rather than whole husk, in starch food models. We included high amylose corn starch for which, to the best of our knowledge, there are no reports on amylose leaching under high temperature (>100 °C). Furthermore, studies about fibre effect on high amylose starches are sparse. F1 fibre used in this work is heteroxylan with low A:X ratio (0.17), for which there are also limited studies. To conclude, this work clearly shows that fibre fractions extracted from the same source can have contrasting effect in starch based gels, which should be taken into consideration when developing new food products.



Supplementary Figure 1. RVA of F1 (a) and F2 (b) at 3 temperature profiles. Blue colour represents RVA curve at 95 °C, green at 120 °C, and purple at 140 °C.

Supplementary table 1. Texture parameters (hardness and adhesiveness) values presented as means of three replicates $\pm$ standard deviation. Different letters correspond to significantly different results at $p < 0.05$.

	95°C			120°C			140°C			95°C			120°C			140°C			120°C			140°C		
	CS	CS+F2	CS+F1	CS	CS+F2	CS+F1	CS	CS+F2	CS+F1	WCS	WCS+F2	WCS+F1	WCS	WCS+F2	WCS+F1	WCS	WCS+F2	WCS+F1	HACS	HACS+F2	HACS+F1	HACS	HACS+F2	HACS+F1
Hardness (g)	91.1±8.7a	78.6±6.2ab	74.2±9.5abc	65.1±3.1bc	84.8±5.4a	74.6±15.2abc	57.1±9.9c	76.9±14.0ab	63.3±13.7bc	/	/	/	/	/	/	/	/	/	42.0±5.4bc	59.9±6.0b	32.7±2.6c	119.4±15.4a	125.7±22.0a	112.8±11.1a
Adhesiveness(g/s)	-46.1±4.5ab	-39.6±10.8a	-67.0±18.8cde	-82.5±5.2ef	-85.9±7.6f	-75.0±7.2def	-58.4±3.3bc	-70.2±2.9cdef	-61.5±3.3bcd	-36.4±1.8a	-44.5±2.9b	-50.8±2.6c	-35.6±3.0a	-44.5±4.7b	-47.9±3.0bc	-32.8±1.5a	-37.8±1.7a	-37.8±2.7a	-35.7±0.9A	-39.8±2.0a	-31.5±1.9a	-78.5±10.2c	-78.7±5.8c	-64.4±6.3b

A) Corn starch

	Constant K	Measured HE	Estimated GI	Leached amylose	Syneresis	Hardness	Adhesiveness	Peak viscosity	Through viscosity	Breakdown viscosity	Final Viscosity	Setback viscosity	Time of peak viscosity	Pasting temperature	Peak temperature
Constant K	1.00														
Measured HE	0.62	1.00													
Estimated GI	0.89	0.91	1.00												
Leached amylose	-0.19	-0.39	-0.29	1.00											
Syneresis	0.13	0.55	0.34	-0.48	1.00										
Hardness	0.19	-0.21	-0.05	-0.52	-0.02	1.00									
Adhesiveness	0.55	0.16	0.36	-0.28	0.27	0.15	1.00								
Peak viscosity	-0.47	-0.20	-0.36	-0.47	0.31	0.44	-0.02	1.00							
Through viscosity	0.55	0.50	0.53	-0.87	0.50	0.45	0.56	0.12	1.00						
Breakdown viscosity	-0.74	-0.56	-0.68	0.58	-0.31	-0.21	-0.54	0.38	-0.87	1.00					
Final Viscosity	-0.04	0.23	0.09	-0.89	0.40	0.52	-0.05	0.55	0.71	-0.39	1.00				
Setback viscosity	-0.52	-0.10	-0.33	-0.51	0.14	0.34	-0.55	0.67	0.14	0.20	0.80	1.00			
Time of peak viscosity	0.68	0.84	0.86	-0.32	0.37	-0.03	0.05	-0.24	0.51	-0.60	0.34	0.04	1.00		
Pasting temperature	0.26	0.02	0.11	0.25	-0.08	-0.16	0.19	-0.68	-0.06	-0.28	-0.51	-0.67	-0.24	1.00	
Peak temperature	-0.23	-0.33	-0.24	0.73	-0.62	-0.48	-0.41	-0.33	-0.66	0.45	-0.46	-0.09	-0.03	-0.28	1.00

B) Waxy corn starch

	Constant K	Measured HE	Estimated GI	Syneresis	Hardness	Adhesiveness	Peak viscosity	Through viscosity	Breakdown viscosity	Final Viscosity	Setback viscosity	Time of peak viscosity	Pasting temperature	Peak temperature
Constant K	1.00													
Measured HE	0.39	1.00												
Estimated GI	0.62	0.96	1.00											
Syneresis	-0.31	-0.12	-0.17	1.00										
Hardness	0.22	-0.10	-0.01	-0.51	1.00									
Adhesiveness	-0.49	0.05	-0.10	0.42	-0.93	1.00								
Peak viscosity	-0.58	-0.38	-0.43	0.10	0.15	0.11	1.00							
Through viscosity	0.05	-0.24	-0.29	-0.06	0.16	-0.31	-0.57	1.00						
Breakdown viscosity	-0.23	0.06	0.08	0.08	-0.07	0.28	0.77	-0.96	1.00					
Final Viscosity	0.56	0.12	0.20	-0.24	0.47	-0.70	-0.56	0.71	-0.74	1.00				
Setback viscosity	0.73	0.35	0.50	-0.29	0.53	-0.74	-0.35	0.24	-0.30	0.85	1.00			
Time of peak viscosity	0.84	0.60	0.74	-0.25	0.02	-0.29	-0.84	0.21	-0.44	0.52	0.57	1.00		
Pasting temperature	0.75	0.40	0.52	-0.12	0.16	-0.42	-0.89	0.46	-0.65	0.70	0.63	0.82	1.00	
Peak temperature	0.85	0.60	0.74	-0.26	0.03	-0.30	-0.83	0.20	-0.43	0.53	0.58	1.00	0.82	1.00

C) High amylose corn starch

	Constant K	Measured HE	Estimated GI	Leached amylose (SB)	Syneresis	Hardness	Adhesiveness	Peak viscosity	Through viscosity	Breakdown viscosity	Final Viscosity	Setback viscosity	Time of peak viscosity	Pasting temperature	Peak temperature
Constant K	1.00														
Measured HE	0.47	1.00													
Estimated GI	0.68	0.97	1.00												
Leached amylose	0.27	0.92	0.84	1.00											
Syneresis	-0.70	-0.23	-0.44	-0.28	1.00										
Hardness	-0.14	0.60	0.37	0.85	0.09	1.00									
Adhesiveness	0.23	-0.57	-0.31	-0.85	-0.12	-0.98	1.00								
Peak viscosity	0.80	0.20	0.39	0.14	-0.58	0.08	0.06	1.00							
Through viscosity	0.10	-0.28	-0.17	-0.50	0.05	-0.59	0.63	0.14	1.00						
Breakdown viscosity	0.37	0.61	0.60	0.88	-0.66	0.96	-0.93	0.25	-0.61	1.00					
Final Viscosity	0.22	0.39	0.39	0.41	-0.62	0.04	-0.03	0.04	0.21	0.37	1.00				
Setback viscosity	0.20	0.50	0.47	0.58	-0.59	0.35	-0.33	0.04	0.04	0.54	0.97	1.00			
Time of peak viscosity	0.18	0.59	0.54	0.37	0.19	-0.55	0.64	-0.16	0.12	-0.01	0.71	0.67	1.00		
Pasting temperature	0.44	0.65	0.66	0.38	0.05	-0.19	0.16	-0.06	-0.25	0.06	-0.16	-0.14	0.34	1.00	
Peak temperature	0.24	0.88	0.78	0.81	-0.10	0.96	-0.93	-0.10	-0.27	0.49	0.64	0.73	0.84	0.44	1.00

Supplementary figure 2. Correlation matrix for starch gels. Green cells represent positive correlation, and red cells represent negative correlation. Bold values represent significant correlation coefficient.

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CHAPTER 7

Application of *P. ovata* and *P. turrifera* fibre in gluten-free bread reveals protective effect on storage stability, but differences in bread quality

Title:

Application of *P. ovata* and *P. turrifera* fibre in gluten-free bread reveals protective effect on storage stability, but differences in bread quality

Authors:

Lucija Strkalj^{1,2}, William J. Hirt², Gleb E. Yakubov¹, Rachel A. Burton², James M. Cowley^{2*}

¹Soft Matter Biomaterials and Biointerfaces, Food Structure and Biomaterials Group, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom

²School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

*Corresponding author

Highlights:

- *Plantago* fibre increased firmness, consistency, and cohesion of GF rice dough.
- *P. ovata* increased the specific volume, loaf crown curvature and improved hardness.
- *P. ovata* and *P. turrifera* fibre improved storage stability of GF rice bread.

Abstract:

The gluten-free (GF) diet, despite its growing prevalence and popularity, is still lower in nutritional value and quality than the conventional, gluten-containing, diet. Extensive research has been conducted on improving GF breads, and one of the approaches is fibre or hydrocolloid addition. This study aims to investigate 1% addition of *Plantago ovata* and *Plantago turrifera* fibre in rice GF bread, while adjusting hydration level for each recipe as per Mixolab measurements. The dough back extrusion showed that fibre increased firmness and consistency. The addition of *P. ovata* fibre resulted in bread with higher specific volume, loaf crown, and improved texture profile analysis, while *P. turrifera* did not show differences from the control GF rice bread. All breads had acceptable crust and crumb colour. Both fibres showed a protective effect on the storage properties, with increased moisture content and decreased hardness than the control bread. We propose that fibre addition slowed down retrogradation, thus improving storage stability of GF rice bread. Addition of *P. ovata* fibre did not change starch hydrolysis parameters from the control bread, while *P. turrifera* decreased hydrolysis extent, but increased hydrolysis rate. The incorporation of *Plantago* fibre exerts a beneficial influence on certain measured properties of the system. Further investigation into the underlying mechanisms is warranted to enhance our understanding of these effects.

Keywords:

Plantago ovata, *Plantago turrifera*, gluten-free bread, bread storage stability, starch hydrolysis

1. Introduction

Gluten-free (GF) diet, which is the only recommended treatment for people with celiac disease, is becoming more popular and prevalent for population with celiac disease, GF intolerance, and people who practice this diet voluntarily (Diez-Sampedro et al., 2019). It is estimated that 1% of world population had celiac disease, but 5% of people in Western countries adhere to GF intentionally (Diez-Sampedro et al., 2019). GF is perceived as “healthier choice”; however, research has shown that it is lower in nutritional value and could be pro-inflammatory (Cappelli et al., 2020; Diez-Sampedro et al., 2019; Henriques et al., 2021; Vici et al., 2016). A systematic review has shown that a GF diet is poor in fibre, certain micronutrients (vitamins D and B12, and folate), rich in saturated fatty acids, and has a higher glycemic index and glycemic load (Vici et al., 2016). In addition to decreased nutritional value, GF bakery and baked goods often have poorer physico-chemical and sensory properties, and shorter shelf-life (Šmídová & Rysová, 2022). Research on sensory perception of GF products in the Hungarian market, for example, has revealed that 70.2% of consumers are not satisfied with quality GF products with products being given an average score of 6.2 out of 10 (Toth et al., 2020). Additives are therefore needed in the product development process to account for poor functionality in native GF flours.

The two most used flours for GF bread preparation are rice and corn, as they are light in colour, are hypoallergenic, and have mild flavour (Foschia et al., 2016). Rice is used in 59.3% of commercial formulations, and the use of corn is 40.7% (Foschia et al., 2016). The disadvantage of using these flours is that their proteins are low in biological value due to the incomplete amino acid composition and cannot form network during mixing and proving. Rice flour proteins are unable to provide structural support and entrap gas during baking, which is important for volume and texture (Foschia et al., 2016). Therefore, different approaches to improve nutritional and functional quality have been proposed, such as inclusion of pseudocereals (quinoa, amaranth, buckwheat, millet), proteins, fibres and hydrocolloid, or adjusting breads' preparation process, such as the use of sourdough (Bender & Schönlechner, 2020; Gobbetti et al., 2018; Šmídová & Rysová,

2022). Fibres and hydrocolloids have been extensively studied in this regard, as they can fulfill multiple roles that lead to the improvement of GF products: improvement of nutritional composition, acting as a network and providing structure, maintaining moisture and storage stability (Culetu et al., 2021). Psyllium husk or *Plantago ovata* husk is one such hydrocolloid that has been utilized to improve GF properties. The first study that has investigated inclusion of psyllium in GF bread was published in 1994, where it was found that the combined effect of 10 g of psyllium and 20 g HPMC (hydroxypropyl methyl cellulose) produced bread with higher specific volume and better hedonic rate (7 out of 9) (Haque & Morris, 1994). Currently, psyllium is gaining scientific popularity, as the number of papers published on the addition of psyllium in GF products has risen in the last years (Culetu et al., 2021). Moreover, it is the third most used substitute in GF baking, after HPMC and xanthan gum (Belorio & Gómez, 2022). Inclusion of psyllium in GF recipe requires adjustment of water addition due to its high water absorption capacity (Mancebo et al., 2015; Ren, Yakubov, et al., 2020). Overall, psyllium protects against moisture loss during storage (Filipčev et al., 2021; Pejcz & Burešová, 2022), acts as gluten network (Mariotti et al., 2009; Ren, Linter, et al., 2020), can increase specific volume (Fratelli et al., 2018, 2021), improves texture (Filipčev et al., 2021), and improves nutritive value and glycaemic index (Fratelli et al., 2018, 2021; Gao et al., 2024; Zandonadi et al., 2009). If, however, the water level is not balanced, its addition can lead to increased baking loss and breads with lower consumer acceptance (Gao et al., 2024; Pejcz & Burešová, 2022).

Previously, the effects of *Plantago* fibre addition on appearance, rheology, and starch hydrolysis behaviour were studied by using simple rice starch-fibre gel composites (Chapter 4). Starch-fibre gels that contained fractions extracted at 25 and 65 °C were acceptable in colour, and all the fractions had a positive effect on rheology. To potentially scale up the extraction process which would be relevant for industry use, combined fraction (extracted only at 65 °C) was used from *P. ovata* and *P. turrifera*. Interestingly, despite a range of literature showing reductions in starch hydrolysis when *P. ovata* husk is added, *Plantago* fractions increased hydrolysis rates, except for the *P. ovata* fraction

extracted at 65 °C. However, these findings may not be transferable to formulated products, as the findings in chapters 3 and 6 are based on simple starch-fibre gels, and so further work was needed to test these behaviors in food products. Therefore, to address these queries, the fibre from *P. ovata* was used due to its beneficial effect on starch hydrolysis, and the fibre from *P. turrifera* was used due to positive changes on viscoelastic profile of rice starch gel and due to its high yield of extracted fibre, which is commercially viable. The scientific aim of this study was to evaluate the effect of each fibre on the structural and quality parameters of GF rice dough and bread, and to look into potential differences of their application.

1% addition of *P. ovata* fibre and *P. turrifera* fibre was added to rice flour GF bread and their hydration level was adjusted with Mixolab to achieve the same consistency as the control rice flour dough. Back extrusion analysis was performed on the doughs, and bread quality parameters, such as volume, colour and texture profile analysis, were obtained. Storage stability after 3 days at room temperature was assessed by moisture loss determination and texture analysis. Finally, the starch hydrolysis by α -amylase was measured to study the potential effects of fibre on starch digestibility of GF bread.

2. Materials and methods

2.1. Materials

White rice flour (Gaganis), table salt (Black & Gold), dry yeast (Tandaco), sugar (CSR), and sunflower oil (Crisco) were purchased in a local supermarket (Foodland, Adelaide, South Australia). Canola oil spray (Woolworths) was purchased in Woolworths (Adelaide, South Australia).

Total starch content of the white rice flour was determined to be $80.0 \pm 2.8\%$ dwb ($70.8 \pm 2.4\%$ as is, 11.42% moisture) using the Rapid Total Starch Method from the K-TSTA amyloglucosidase/ α -amylase kit (Megazyme, Ireland).

P. ovata and *P. turrifera* seeds were originally sourced and produced as per Cowley et al. (2021), and where applicable, collection complied with relevant institutional, national, and international guidelines and legislation.

Porcine pancreatic α -amylase (E-PANAA) was purchased from Megazyme (Neogen Australasia, Queensland). D-maltose monohydrate (63418), 4-hydroxybenzhydrazide (H9882), sodium carbonate (223530), and sodium phosphate monobasic (S0751) were purchased from Sigma Aldrich (New South Wales). Sodium phosphate dibasic (SA026) and sodium hydroxide (SA000) were purchased from ChemSupply (South Australia). Hydrochloric acid (RP1104) was purchased from RCI Labscan (Victoria).

Abbreviations used in the remainder of the text are: CRB = control rice bread, POB = bread with *P. ovata* fibre, PTB = bread with *P. turrifera* fibre.

2.2. Methods

2.2.1. Fibre extraction

For fibre extraction, 20 g of *P. ovata* seeds or 10 g of *P. turrifera* seeds (adjusted based on their water absorption capacities) were placed in 800 mL of RO H₂O. The mixtures were stirred with a magnetic stirrer for 4 h at 65 °C. After heating, the mixture was strained through a fine kitchen sieve (0.25 mm pores) was placed in an oven and dried (12 h, 95 °C) to obtain fibre. After drying, fibre was ground using a coffee grinder (Sunbeam, Australia) and sieved through a 200 μ m sieve (Mesh 80, Haines Educational, Wantirna South, Australia). Fibre powders were stored in a closed plastic container until the breadmaking process.

2.2.3. Adjusting the hydration level of the dough

To avoid the effect of water deficit, control bread and fibre-added breads were prepared with varying water content to achieve equivalent dough consistencies as measured by Mixolab 2 (Chopin, France).

Preliminary baking experiments were performed using the control formulation with varying water contents, and the optimum hydration for the control was determined empirically based on resultant bread quality (i.e. some were too liquid to prevent total

collapse during proofing and some were too firm to rise during proofing at all). To determine the optimum dough consistency target for the fibre-added formulations, an equivalent control dough, minus the sunflower oil and yeast, was also prepared using the Mixolab 2 and the Chopin S protocol modified to use 90 g of dough. The average torque between 4 and 10 minutes was selected as the target consistency to be used for the fibre-added test formulations. Table 1 shows the baking and Mixolab 2 formulas that were tested, along with the hydrations calculated for each and the resulting torques from the Chopin S runs.

Table 1. Ingredients list, moisture content, and dough formulas for each bread. Overall hydration (%) for each bread is expressed, as well as average torque (Nm) for minute 4-10 as recorded by Mixolab. Each dough recipe is presented as weight of ingredients (g), ratio (%) of ingredients in the dough, and ratio (%) of ingredients used for Mixolab run (flour, sugar, salt, and fibre if included).

		Control Rice Flour Dough			Rice Flour Dough with <i>P. ovata</i> fibre			Rice Flour Dough with <i>P. turrifera</i> fibre		
		CRF			POD			PTD		
Ingredient	Ingredient Moisture Content (%)	Baking Ingredient Weights (g)	Baking Formula (%)	Mixolab Formula (%)	Baking Ingredient Weights (g)	Baking Formula (%)	Mixolab Formula (%)	Baking Ingredient Weights (g)	Baking Formula (%)	Mixolab Formula (%)
Rice Flour	11.42	100.00	50.80	53.98	100.00	46.26	49.14	100.00	46.70	49.59
Sugar	0.40	5.00	2.54	2.70	5.00	2.31	2.46	5.00	2.34	2.48
Salt	0.05	2.00	1.02	1.08	2.00	0.93	0.98	2.00	0.93	0.99
Sunflower Oil	9.00	5.00	2.54	---	5.00	2.31	---	5.00	2.34	---
Yeast	4.60	1.50	0.76	---	1.50	0.69	---	1.50	0.70	---
<i>P. ovata</i> fibre	9.70	---	---	---	1.00	0.46	0.49	---	---	---
<i>P. turrifera</i> fibre	9.05	---	---	---	---	---	---	1.00	0.47	0.50
Water	100.00	83.35	42.34	42.24	101.68	47.03	46.93	99.61	46.52	46.44
Total		196.85	100.00	100.00	216.18	100.00	100.00	214.11	100.00	100.00
Moisture Content of Formula (%)			48.4			52.6			52.2	
Dry Matter Content of Formula (%)			51.6			47.4			47.8	
Hydration (%)			93.9			111.0			109.0	
Average Torque from 4-10 Minutes (Nm)			0.176			0.176			0.180	

CRF = control rice flour dough, CRB = control rice flour bread, POD = dough with *P. ovata*, POB = bread with *P. ovata*, PTD = dough with *P. turrifera*, PTB = bread with *P. turrifera*

Due to sample limitations of the fibre fractions, iterative Chopin S protocols to determine the hydrations required to achieve the target torque were not practical. Instead, a custom protocol was used to home in on the target hydration in a single run through incremental water additions. Due to the strong water absorbing properties of the fibre,

the initial hydration level for dough with *P. ovata* fibre was taken to be the same as the control, or 93.9%. Throughout the run, the dough was mixed at a controlled temperature of 30 °C and controlled mixing speed of 80 rpm. After the initial water addition, the dough was first mixed for four minutes to stabilise the hydration and torque. At four minutes, 0.5 g of water was added through the manual addition function on the Mixolab 2. Each minute after that, another 0.5 g of water was added until the torque read significantly below the target torque of 0.176 Nm. This experiment was conducted again to refine the hydration by beginning with an initial hydration level closer to the actual target level. Finally, a confirmation run (modified Chopin S protocol using 90 g of dough) was conducted to confirm that the hydration level (this time added in a single step) was appropriate to achieve the target torque ($0.176 \pm 3\%$).

Since it was assumed that dough with *P. turrifera* fibre would behave more similarly to dough with *P. ovata* fibre than to the control dough, the starting hydration chosen for the *P. turrifera* step-down experiment was higher, 105% instead of 93.9%. Because the experiment started much closer to the target torque level, it was not required to repeat the experiment to refine the hydration, thus the experiment was followed directly by confirmation runs.

2.2.4. Bread preparation and baking

Weights of each ingredient used in breadmaking are specified in Table 1. Rice flour, fibre (if added), sugar, salt, yeast, water, and oil are mixed with tilt-head stand mixer (KSM195, KitchenAid, South Australia). After mixing for 10 min at speed 2, 100 ± 1 g of dough was placed in pup loaf pans (5.5×5.0×10.0 cm) pre-oiled with canola oil spray. Doughs were then placed in proofing oven (Turbofan P85M12, Moffat, Australia) at 37 °C, 80% moisture for 45 min. Doughs were then baked at 180 °C for 20 min (convection steam oven, COMBI-plus, Hobart, New South Wales, Australia). After baking, breads were left to cool at room temperature for 1 h.

2.2.5. Dough back extrusion

A portion of 50±1 g of dough was placed in the acrylic cylinder for back extrusion (50 mm radius). The dough was placed carefully, to ensure there were no air pockets which would disturb the measurement. A texture analyser (TA.XTplusC, Stable Micro Systems, United Kingdom) with 40 mm back extrusion rig (A/BE, Stable Micro Systems, Australia) was used. Triggering force was 1 g, pre-, mid-, and post-test speeds were 20 mm/s, penetrating to the sample depth of 10 mm after which the probe returned to original position (45 mm). Exponent Connect version 8.0.17.0 was used for data collection, and firmness, consistency, and cohesiveness were calculated.

2.2.6. Bread appearance and properties determination

Baking loss was determined by weighing the loaves after cooling at room temperature for 1 h and using Equation 1 for calculation.

Equation 1:

$$\text{Baking loss (\%)} = \frac{(\text{Mass of dough before baking} - \text{mass of bread after cooling for 1 h})}{\text{Mass of dough before baking}} * 100$$

The volume of bread was determined by rapeseed displacement method AACC 10-05.01 (AACC, 2010).

Specific volume was calculated according to Equation 2.

$$\text{Equation 2: Specific volume } \left(\frac{\text{mL}}{\text{g}}\right) = \frac{\text{loaf volume (mL)}}{\text{loaf mass (g)}}$$

The breads were sliced to 1.5 cm thickness with an electric rotary blade slicer. Photo of slices was taken at constant distance with a digital camera (MIUI Global 14.0.11, China). Moisture was determined according to AACC international method 44-15.02 two-stage bread moisture determination with second step adjustment (AACC, 1999). Briefly, bread slices were cut in pieces (approximately 1 cm length and height) and were let to air dry overnight. Next day, pieces were ground using coffee grinder and sieved through 200 µm sieve. Breadcrumbs (3 g) were placed into aluminum pans in a moisture analyzer (MB120,

Ohaus, Germany) and moisture content was analysed as per manufacturer's instructions and calculated according to Equation 3.

$$\text{Equation 3: } \textit{Moisture content (\%)} = A + \frac{(100-A)*B}{100}$$

A is moisture content determined by the first step (air drying), and B is moisture determined by the second step (moisture analyzer).

Loaf height:width ratio and loaf crown curvature (LCC) indices were calculated from the bread slice images by image analysis using Fiji ImageJ (Schneider et al., 2012). Loaf height to width was calculated as the ratio between the mid-slice height and the maximum loaf width. LCC index was calculated according to Equation 4.

Equation 4:

LCC index

$$= \frac{\textit{Maximum loaf width (shoulder to shoulder) (mm)}}{\textit{Perimeter of the top of the slice from shoulder to shoulder (maximum width)(mm)}}$$

The colour of the crust and crumb was determined using (Colorflex EX, HunterLab, Reston, United States) after calibration with white and black ceramic tile before each run. Total colour difference (ΔE^*) of the fibre-added breads was calculated relative to the control bread using the ColorTools add-in for MS Excel (<http://rgbcmyk.com.ar/en/xla-2/>).

2.2.7. Texture profile analysis of bread

A texture analyser (TA.XTplusC, Stable Micro Systems, United Kingdom) fitted with a 5 kg load cell and 36 mm compression cylinder probe (P/36R, Stable Micro Systems, UK) was used for texture profile analysis (TPA). Using a cylindrical cookie cutter (2.5 cm radius), representative was used to cut portions of crumb from the bread slices (1.5 cm thickness). The triggering force was 1 g and pre-, mid-, and post-test speeds were 20 mm/s. The depth of compression was 7 mm (<50% compression) after which the probe returned to original position, and compression was repeated. Exponent Connect version

8.0.17.0 was used for data collection, and hardness, chewiness, cohesiveness, and adhesiveness were calculated using the standard simplified TPA macro.

2.2.8. Storage stability

Prepared crumb parts (as described in previous section, 1.5 cm height, 2.5 cm radius) from six slices were weighed on Day 0 (day of bread baking), placed in plastic freezer bags, and left at room temperature for 3 days without opening. After 3 days, the bags were opened and bread pieces were weighed again. Storage loss was calculated according to Equation 5.

$$\text{Equation 5: Storage loss (\%)} = \frac{(\text{Mass of bread at day 0} - \text{mass of bread at day 3})}{\text{Mass of bread at day 0}} * 100$$

To assess the potential effect of fibre on storage stability, texture profile analysis was repeated on bread pieces which were stored for 3 days. TPA was performed as described in previous above (Section 2.2.6.) though to account of the substantial increased in crumb firmness, the samples were compressed to 2.5 mm. Hardness was calculated using Exponent Connect version 8.0.17.0. Staling estimated as the change in hardness after storage using Equation 6:

Equation 6:

$$\Delta \text{Hardness (\%)} = \frac{(\text{Force at 25 mm on first compression (Day 3)} - \text{Force at 25 mm on first compression (Day 0)})}{\text{Force at 25 mm on first compression (Day 0)}} * 100$$

2.2.9. Starch hydrolysis by α -amylase and PAHBAH assay for determination of released maltose

α -amylase starch digestibility assay was done according to Edwards et al. (2019). Starch to α -amylase ratio is kept constant in all samples, thus varying amounts of bread were calculated to 100 mg of starch base and corrected for moisture. Ground bread (crumbs smaller than 200 μm) was placed into 15 mL tubes and suspended in 10 mL phosphate buffered saline (pH=7.4). After brief homogenization (PROScientific Premium MaX homogenizer, PRO Scientific Inc., CT, USA) sample tubes were placed in blood rotator in incubator (Bioline, Edwards Instrument Company Australia, New South Wales) at 37 °C

and equilibrated for 30 min. Prior to reaction, 100 μL of each sample was transferred into 1.5 mL microcentrifuge tube which contained 100 μL of 0.3 M Na_2CO_3 ("stop solution"). Stop solution inactivates the enzyme and stops the reaction at each time point. To start the enzymatic reaction, porcine pancreatic α -amylase was added. Enzyme was carefully weighed before the start of the experiment, so the enzyme activity was 5.5 AU/mL, and dissolved in PBS. Immediately after the addition of the enzyme, tubes were returned into the incubator and rotated at 37 $^{\circ}\text{C}$ for the rest of the experiment. At pre-defined time points (2, 5, 7, 10, 15, 30, 90, 120, 150, 180 min) 100 μL aliquots were taken from the reaction solution and placed into microcentrifuge tubes with equal amount of stop solution. After brief vortexing, all tubes were centrifuged at 15 000 g for 5 min (Eppendorf centrifuge 5417R, New South Wales) to exclude any starch remains and 100 μL of supernatant was placed into new microcentrifuge tubes, which were then used to determine the amount of reducing sugars.

Reducing sugar analysis (PAHBAH assay) was used to determine the amount of reducing sugars which are the product of α -amylase reaction at each sampled time point (Lever, 1972). Supernatants were diluted (1:20) in water, and 100 μL of the diluted samples was placed in heat-safe microcentrifuge tube which contained 1 mL of PAHBAH reagent (preparation: 250 mg of p-hydroxybenzoic acid hydrazide dissolved in 4.75 mL of 0.5 M HCl and 45 mL of 0.5 M NaOH). The mixture is vortexed, and then placed in boiling water bath (100 $^{\circ}\text{C}$) for 5 min. After cooling to room temperature (15 min), 300 μL of each solution was transferred to a well in 96-well plate with flat bottom and the absorbance was measured at 405 nm (μQuant , Bio-tek instruments, Queensland). Standards containing known concentration of maltose (concentrations ranging from 100 μM to 1000 μM) were prepared the same way as described above and used for creating the calibration curve against which the samples were measured.

2.2.10. Statistical analysis

Data is presented as mean and standard deviation of at least triplicates. Starch hydrolysis curves were fitted in GraphPad Prism 9.0.0. software. Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD

post-hoc test ($p < 0.05$). Graphs were made in GraphPad Prism 9.0.0. software, unless otherwise stated.

3. Results and discussion

3.1. Dough properties: hydration level and back extrusion

When psyllium fibre is added into GF (or gluten-containing) bread, adjustment of hydration addition is needed to compensate for its high water holding capacity. Otherwise, if the water addition level is too low, it might cause gas cell collapse, dense crumb structure, and high dough rigidity. Conversely, if the water level is too high, it might cause weak crumb and dough instability (Ren, Linter, et al., 2020). In this work, Mixolab 2 was used to determine the water addition necessary to obtain each formulations with the appropriate dough consistency. Through careful adjustment of water addition, a target torque of 0.176 Nm was achieved for each formulation (Fig. 1).

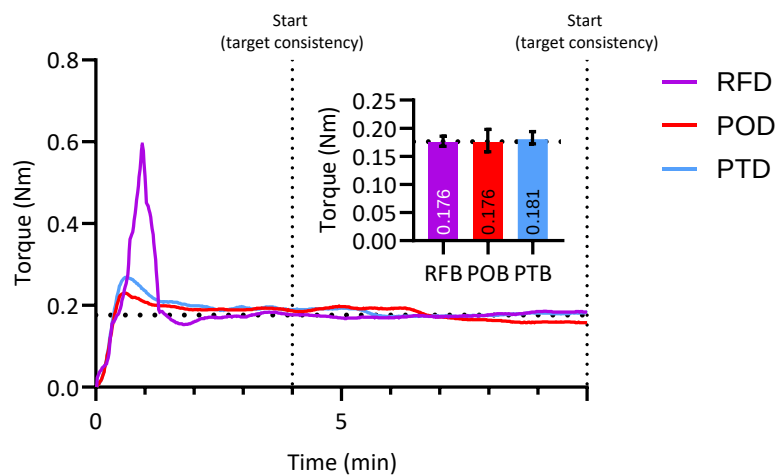


Figure 1. Mixolab curves of rice flour dough (RFD), dough with *P. ovata* fibre (POD), and dough with *P. turrifera* fibre (PTB). Target torque was 0.176 Nm, which is marked with dotted line on y-axis. This target achievement was observed from min 4 until min 10,

which is also marked by dotted lines on x-axis. The inset summarizes the mean and range of torque in the 4-10 min window.

Based on rice flour, salt, sugar, and fibre (if added) hydration for rice flour dough (RFD) was 93.9%, for the dough containing *P. ovata* fibre (POD) was 111.0%, and for dough containing *P. turrifera* fibre (PTB) was 109.0%. Interestingly, to achieve the same consistency a slightly lower amount of water was added for PTB, even though *P. turrifera* fibre has higher water absorption capacity than *P. ovata* fibre (121.95 and 72.13 g water/g fibre, Chapter 3, Fig. 1B). However, the difference could be attributed to the innermost mucilage layer, rather than fractions extracted here (at 65 °C, while the innermost layer is extracted via intense shaking, Chapter 4, Section 2.1.). However, this could also indicate that in a complex mixture, such as bread dough, ingredients might act differently than when they are examined individually. Also of note is the large spike in torque at 1 min for RFD that is not present in POD and PTD, despite stabilizing at comparable torques soon after. We assume that this is related to granule-granule friction of rice flour which is dampened when psyllium viscosifies the intergranular water. Mariotti et al. (2009) studied psyllium in gluten free bread (corn starch, amaranth flour, and pea isolate as the base) and stated that at a 2% replacement level and with additional added water, psyllium improved workability and provided more cohesive network in the gluten-free dough. They showed that even when consistency of different dough blends is constant (200 BU), rheological fundamental properties can vary, as the dough was less frequency dependent when psyllium was added (Mariotti et al., 2009). Gluten-free doughs are known to be difficult to work with, since it can resemble more of liquid-like batter, rather than workable dough. Cappa et al. (2013) have studied the influence of psyllium, sugar beet fibre and different consistencies (200 or 500 BU) by varying the water addition on gluten free bread. They reported that lower consistency, which was pourable but not workable, produced breads with higher quality specifically when ingredients with high hydration capacity (such as psyllium) were included in the recipe (Cappa et al., 2013). However, other authors report that increased dough workability provides structure which is more gluten-like (Pejcz & Burešová, 2022). The recipe

presented in this work (Table 1) produced pourable doughs (not shown) which resembled a batter, rather than a dough. An important discerning factor to consider is the fact that the isolated fibre fractions were used in this work, whereas in the majority of published research the whole psyllium husk is used, there is an insoluble fibre component as well. Potentially, this could have also had an influence on suitability of dough with lower consistency.

Back extrusion measurement provided useful parameters regarding the dough structure (Table 2) and its flowability.

Table 2. Back extrusion parameters of doughs. CRB – control rice bread, POB – bread with *P. ovata* fibre, PTB – bread with *P. turrifera* fibre. Values are presented as mean of three replicates $\pm$ standard deviation. One way ANOVA with post-hoc Tukey test was used to determine statistically significant difference ($p < 0.05$) between samples and marked with different letters.

	Firmness	Consistency	Cohesiveness
CRB	311.49 $\pm$ 17.57c	2398.71 $\pm$ 157.19a	-216.99 $\pm$ 11.34a
POB	464.86 $\pm$ 19.81a	2678.62 $\pm$ 180.95a	-271.45 $\pm$ 14.56b
PTB	395.99 $\pm$ 18.17b	2625.05 $\pm$ 355.29a	-242.14 $\pm$ 11.20a

Firmness and consistency are impacted by water addition level, and they can be used to predict final products' quality. Cohesiveness represents structural integrity of the dough and is often defined by the resistance of a material to being pulled apart. A negative measurement indicates that a higher force was required to withdraw the probe from the material, suggesting stronger internal bonding. This is typically measured during a backward extrusion test, where the maximum negative force reflects the cohesiveness of the sample. The results agree with hydration level of the doughs. RFD had the lowest firmness, consistency and cohesiveness (311.49, 2398.71, and -216.99, respectively), and POD the highest (464.86, 2678.62, and -271.97, respectively). PTD's parameters were

slightly lower than those of POD (395.99, 2625.05, and -242.14, respectively). This means that the addition of *P. ovata* fibre resulted in the dough with the highest level of dough stability when subjected to back extrusion test. This finding is contrary to rheology results presented in Chapter 3, (Section 3.5., Fig. 5) where *P. turrifera* fibre showed higher resistance to deformation. For bread baking experiments, we extracted fibre at 65 °C (one step extraction) which we suggest is similar to a pooling of cold water (C) and hot water (H) fractions from Chapter 3 (extracted at 25 and 65 °C, respectively). Fraction C from both *P. ovata* (PO-C) and *P. turrifera* (PT-C) were viscoelastic fluids with similar $\tan \delta$ and A' parameters (PO-C = 0.20, PT-C = 0.26, not significantly different, Chapter 3, Table 1). However, *P. ovata* and *P. turrifera* H fractions (PO-H and PT-H) did show differences: PO-H had lower G' (10.23 and 13.51 Pa, respectively) and higher G'' than PT-H (6.23 and 3.18 Pa, respectively). Correspondingly, PO-H had a higher $\tan \delta$ than PT-H (0.610 and 0.236, respectively). Lastly, PO-H had a far lower flow point than PT-H (12.42 and 80.45 Pa, respectively). All these parameters indicate that PO-H had more pronounced viscous behavior and flowed more easily, i.e. at lower disturbance, thus we would expect lower firmness, consistency and cohesion in materials containing this fraction compared to those containing PO-H. However, as mentioned, rheology results of a single ingredient might not translate in complex mixture and particularly the dilution effect within a matrix must be considered. For example, the ability of psyllium to form films around starch granules (Mariotti et al., 2009) might have contributed to increased back extrusion parameters. To fully understand changes in dough structure when fibres are added, confocal microscopy of stained dough would be useful, as well as full Mixolab profile where dough rheology can be measured dynamically over a simulated baking cycle (Franco et al., 2020).

3.2. Appearance and texture profile analysis of bread

Appearance, quality, and texture parameters of gluten-free breads baked with *Plantago* fibre can be found in Fig. 2 and Table 3.

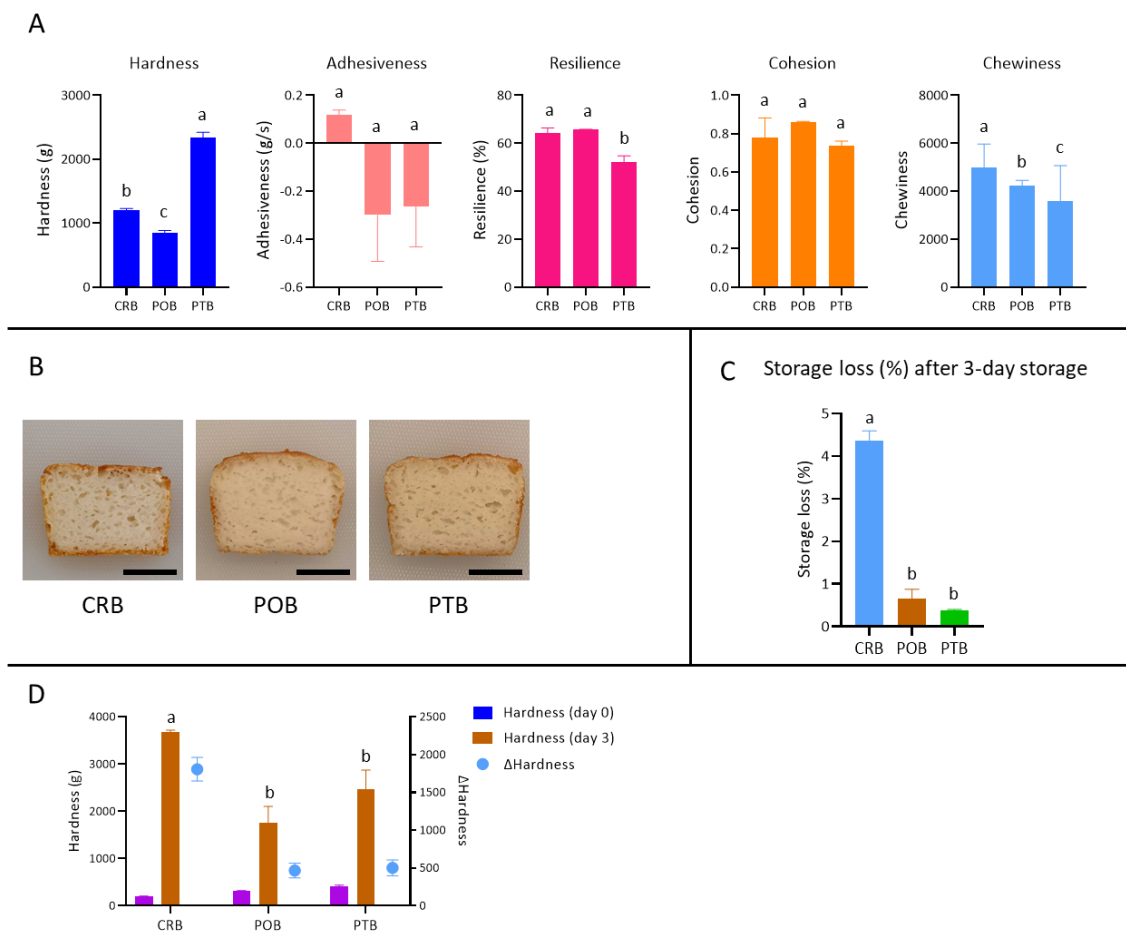


Figure 2. The appearance and texture behaviour of breads. Figure 2A shows texture analysis parameters (hardness, adhesiveness, resilience, cohesion, and chewiness measured at day 0 (day of baking, after 1h of cooling at room temperature). Figure 2B Shows appearance of a slice of each bread. Figure 2C shows storage loss (%) parameter after 3 days of storage. Figure 2D shows hardness at day 0 and day 3 (left y-axis) and difference in hardness after 3-day storage (right y-axis). CRB – control rice bread, POB – bread with *P. ovata* fibre, PTB – bread with *P. turrifera* fibre. Different letters note significantly different means at $p < 0.05$.

Table 3. Quality and colour parameters of breads (CRB – control rice bread, POB – bread with *P. ovata* fibre, PTB – bread with *P. turrifera* fibre). LCC index – loaf crust curvature

index, L* - lightness, a* - greenness to redness parameter, b* - blueness to yellowness parameter. Values are expressed as mean of three replicates $\pm$ standard deviation. Different letters represent statistically significant difference between samples, as determined by one-way ANOVA ($p < 0.05$).

		CRB	POB	PTB
Baking behaviour and bread properties	Baking loss (%)	15.51 $\pm$ 0.04b	16.47 $\pm$ 0.42a	16.43 $\pm$ 0.40a
	Moisture (%)	30.82 $\pm$ 0.75c	37.28 $\pm$ 1.05a	34.40 $\pm$ 0.57b
	Volume (mL)	125.30 $\pm$ 5.11ab	131.10 $\pm$ 4.43a	113.66 $\pm$ 7.22b
	Specific volume (mL/g)	1.48 $\pm$ 0.05ab	1.57 $\pm$ 0.05a	1.35 $\pm$ 0.09b
	Height:width ratio	0.85 $\pm$ 0.01ab	0.89 $\pm$ 0.00a	0.80 $\pm$ 0.06b
	LCC index	0.09 $\pm$ 0.02b	0.19 $\pm$ 0.02a	0.12 $\pm$ 0.03b
Bread colour	Crust L*	32.00 $\pm$ 1.86b	35.84 $\pm$ 0.16a	33.02 $\pm$ 1.63ab
	Crust a*	13.67 $\pm$ 1.83a	12.08 $\pm$ 0.31a	13.40 $\pm$ 0.55a
	Crust b*	34.11 $\pm$ 1.67a	33.33 $\pm$ 0.60a	33.30 $\pm$ 0.20a
	ΔE^*	-	3.29	2.50
	Crumb L*	57.40 $\pm$ 2.19a	57.43 $\pm$ 0.31a	52.54 $\pm$ 1.24b
	Crumb a*	0.35 $\pm$ 0.20b	0.47 $\pm$ 0.02b	1.57 $\pm$ 0.28a
	Crumb b*	17.43 $\pm$ 0.38a	16.10 $\pm$ 0.25b	17.55 $\pm$ 0.35a
	ΔE^*	-	0.78	4.82

Baking loss was increased when fibres were added, however POB and PTB still had higher moisture content than CRB (37.28, 34.40, 30.82%, respectively). This is expected, since more water was added when preparing POB and PTB (Table 3) and the addition of psyllium to bread formulations generally increases the dough hydration due to its high water absorption capacity. For example, psyllium husk (3-9%) decreased the baking loss of rice bread though there was no difference between levels of addition (Pejcz & Burešová, 2022). However, other studies have shown that while psyllium enhances

moisture retention in bread, it does not significantly affect baking loss or weight loss during baking. Gao et al. (2024) have studied varying levels of psyllium replacement (0, 5, 10, 15, 20%) in buckwheat bread and found that increased psyllium proportion also increased baking loss, which was hypothesized to be linked to the porous structure formed by psyllium. Similar to our results, specific volume was increased when psyllium was added (Gao et al., 2024). Both the specific volume and height:width ratio of POB had the highest value when compared to CRB and PTB, but they were not significantly different from CRB (Table 3). Loaf crown curvature (LCC) index was the highest in POB and it visibly has a more prominent rounded top than CRB and PTB (Fig. 2B), which is a positive appearance attribute. Fratelli et al. (2021) also reported increased specific volume and height:width ratio which they attributed to higher dough consistency. Considering that back extrusion experiments discussed in previous section (Table 3) did not show significant difference between CRB and breads with added fibre, we do not believe this explains our results shown here. However, in gluten-free baking, psyllium provides elasticity and extensibility that can mimic some of gluten's properties, allowing the dough to rise properly without collapsing. This is crucial for achieving the desired texture in gluten-free breads, which often have insufficient structural integrity. It can also be noted that the addition of *P. turrifera* fibre had a somewhat negative effect on volume, specific volume, and height:width ratio (Table 3). These changes were not statistically significantly different from CRB, but they were statistically significantly different from POM.

Bread colour parameters (L^* , a^* , b^*) for crust and crumb of the three breads are shown in Table 3. L^* represents darkness to lightness (from 0 to 100), a^* represents greenness to redness (from -128 to +127), and b^* represents blueness to yellowness (from -128 to +127). Overall, all breads were similar in colour, which can also be seen in Fig. 2B. POB had a somewhat lighter crust colour than CRB (35.84 and 32.00, respectively). PTB had slightly darker crumb colour than CRB and POB (52.52, 57.40, 57.43, respectively). There was no difference in a^* and b^* parameters of the crust. PTB crumb showed slightly redder hue ($a^*=1.570$), and POB crumb showed slightly yellower hue ($b^*=16.10$). Overall,

ΔE^* of both crust and crumb were below 5 meaning that the total colour differences are unlikely to be unacceptable colour changes to consumers. Psyllium husk has light beige colour, but at higher addition or replacement content, and due to heat-related pigment changes during baking, it can negatively affect the colour of the bread. The replacement of rice flour with psyllium (25-100%) in gluten-free bread rolls resulted in significant reduction of L^* parameter of the crust (from 83.87 for control bread roll to 70.67 for bread roll with 25% psyllium) (Hastaoğlu et al., 2024). Psyllium had the same effect in buckwheat bread, where 5% replacement decreased crust L^* parameter from 67.81 to 58.03 (Gao et al., 2024).

Texture profile analysis parameters are shown in Fig. 2A. Overall, POB had improved texture over CRB: lower hardness (849 g as compared to 1196 g for CRB) and lower chewiness (4243 as compared to 5461 for CRB). There was no difference in resilience, adhesiveness and cohesion between CRB and POB. Published literature has shown that psyllium usually has positive effect on texture if the hydration level is adjusted, whereas if hydration is constant, then psyllium addition can increase hardness (Ren, Linter, et al., 2020). Considering that hydration level in this work was adjusted, the decrease in hardness is expected and desirable. Cappa et al. (2013) have also reported the lowest hardness for bread with psyllium which had higher water content and whose dough consistency was liquid-like. In another study, the lowest crumb firmness and highest acceptability was reported for bread with increased psyllium addition (7.14 and 17.14%) and increased water addition (91.10 and 117.85%) (Fratelli et al., 2021). Interestingly, even though hydration level was adjusted for *P. turrifera* fibre as well, PTB had significantly higher hardness than CRB and POB (2321, 1196 and 849, respectively). PTB also had the lowest chewiness (3081, as compared to 5461 for CRB and 4243 for POB). Overall, it seems that *P. ovata* fibre had beneficial effect on CRB, but *P. turrifera* fibre did not show the same outcomes. Back extrusion parameters showed that dough with *P. turrifera* had lower firmness than dough with *P. ovata* (395 and 464, respectively) and the lower dough firmness could have led to collapse during baking and denser and more packed crumb, which would result in higher hardness (Culetu et al., 2021; Šmídová &

Rysová, 2022). Perhaps different mechanisms drive behaviors of dough and bread, depending on which fibre is included. Moreover, fibres could exhibit varying viscoelastic profiles when heated at high temperature. Further studies on dough rheology, including temperature sweep, and analysis of dough leavening could provide insight into dough development and, consequently, bread quality.

3.3. Storage stability

Storage stability parameters are presented in Fig. 2C and 2D. The addition of fibres significantly decreased moisture loss during the 3-day storage. CRB, which showed a 4.35% loss in mass to moisture, consequently had the highest hardness after storage (3946 g). Conversely, breads with the added fibre had a lower moisture loss (POB = 0.64%; PT = 0.39%) and were softer than CRB (POB = 1856 g and PTB = 2530 g). While PTB was 36.30% firmer than POB after storage, the difference was not significant. Furthermore, even though PTB at day 0 had the highest hardness, it showed an equally protective effect on storage stability to *P. ovata* fibre (Δ Hardness of POB was 468.6, and PTB was 502.1%). The protective effect of soluble fibres and hydrocolloids on staling is attributed to their high water absorption capacity (WAC). When 2.86% psyllium was added to rice flour and cassava starch gluten-free bread, storage stability also increased (Fratelli et al., 2021). Like the results shown here, there was no storage loss, and crumb firmness was significantly lower than in the control bread. The authors also measured acceptability of aroma, flavor, and texture of the bread after 3 days, and bread with psyllium had higher scores than the control bread (Fratelli et al., 2021). When compared to xanthan gum, HPMC, and guar gum, bread containing psyllium had the highest moisture content after 10 days of storage and it showed the least changes in moisture content (Filipčev et al., 2021). Interestingly, when 3% psyllium husk was compared to 3% whole seed flour from *P. ovata* and *P. psyllium*, whole *P. psyllium* seeds showed the most favorable effect and decreased hardness of bread after 24 h storage (Pejcz & Burešová, 2022). In addition to psyllium's ability to retain water, Mariotti et al. (2009) showed that its ability to form film around corn starch granules had also contributed to slower retrogradation and, therefore, better storage stability. As for CRB, back extrusion

showed that it had the lowest firmness (311.49 g) and consistency (2398 g, although it was not statistically significant), and it was shown that if the dough is too soft initially, it may undergo rapid retrogradation post-baking, leading to a firmer texture as it cools and stales more quickly (Culetu et al., 2021; Šmídová & Rysová, 2022).

3.4. Starch hydrolysis by α -amylase

To further assess the impact of fibre on GF rice bread, the starch hydrolysis by α -amylase is measured and shown in Fig. 3. POB was not significantly different from CRB, both in terms of the hydrolysis rate (0.045 and 0.042 min^{-1} , respectively) or the extent (55.80 and 60.92% , respectively), even though hydrolysis extent was decreased. Interestingly, PTB had a significantly higher hydrolysis rate (0.067 min^{-1}), but decreased hydrolysis extent overall (54.16%). This could potentially indicate an increase in resistant starch, which is beneficial for gastrointestinal health, though this would need to be confirmed quantitatively.

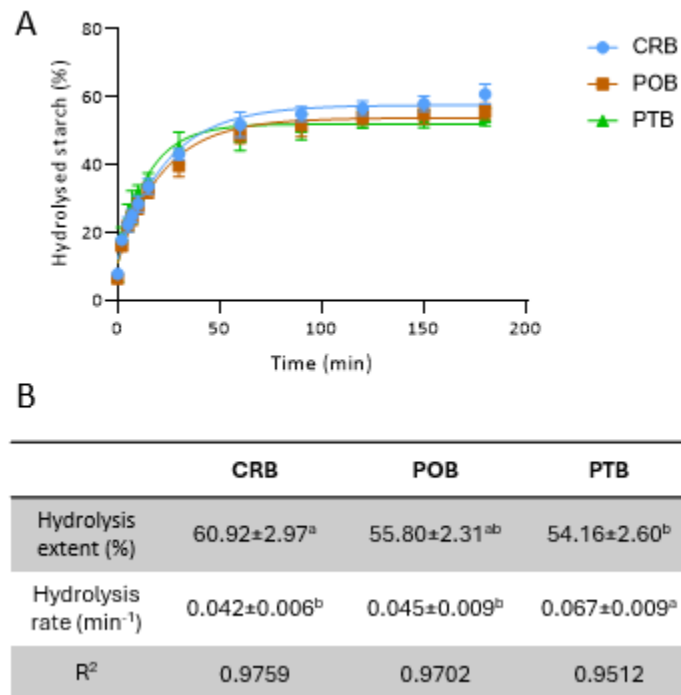


Figure 3. Starch hydrolysis by α -amylase of CRB, POB and PTB. Figure 3A. Starch hydrolysis curves. Figure 3B. Starch extent and rate. Data is presented as mean of three replicates $\pm$ standard deviation. Different letters represent significantly different means at $p < 0.05$.

Research exploring the addition of psyllium in GF and gluten-containing bread has mainly shown positive outcomes, meaning lower starch digestibility. Psyllium compared to xanthan gum and lambda-carrageenan resulted in the highest reduction of glycemic index of white bread (Yassin et al., 2022). Psyllium addition in wheat crackers and bread (replacement of wheat flour by 10%) reduced rapidly digested starch significantly, while cellulose had no significant effect (Bilgic & Sensoy, 2023). The authors contributed this to psyllium's ability to act as a physical barrier around starch granules and restriction of enzyme mobility. Gao et al. (2024) have reported that 5% of psyllium decreased buckwheat starch hydrolysis rate, but not extent. In the same study, higher proportions of psyllium (10, 15, 20%) *decreased* hydrolysis extent, but *increased* hydrolysis rate. The viscosity measurement of digesta revealed that higher psyllium addition led to higher viscosity, as expected, but changes in viscosity did not explain increase in hydrolysis rate (Gao et al., 2024). Overall, it seems that presence of *Plantago* fibres in complex mixture that undergoes mixing, proving, baking, and cooling should be further explored to fully elucidate mechanisms that govern the starch digestibility. In this work, results could be explained by the fact that amount of water was increased according to the added fibre so most likely there is no occurrence of gelatinization reduction, which would reduce starch digestibility potential, and differences in retrogradation, as this experiment was conducted one day after baking. The addition of fibre decreases starch retrogradation, which is also confirmed by storage studies (storage loss and hardness measured after 3-day storage, Section 3.3., Fig. 2C and 2D). Other authors have shown that starch that is retrograded is also less digestible (Patel et al., 2017; Wang et al., 2015), therefore this proposed explanation is probable in the system shown here.

As mentioned in the introduction, one of the aims was to study *P. ovata* and *P. turrifera* fibre in a more complex system (such as GF bread) and assess whether insights gained in

Chapter 4 regarding starch hydrolysis in simple rice starch-fibre gel could be transferable here. To reiterate, fibre fraction used in this chapter is extracted at 65 °C, and represents a mixture of fractions C and H from chapter 4, where fraction C was extracted at 25 °C and fraction H at 65 °C. Both fractions did not have a significant impact on hydrolysis extent (Chapter 4, Fig. 5), which corresponds to the result seen here. As for hydrolysis rate, fraction C slightly, but not significantly, increased the rate, while fraction H decreased it. In POB, we did not see any changes. As for *P. turrifera*, it significantly increased the hydrolysis rate, which agrees with results measured when C and H fractions are added to rice starch (Chapter 4, Fig. 5). PTB has decreased hydrolysis extent which, however, is slightly different from previously shown results, where C fraction increased the extent, and H fraction did not change the extent. This representation may be an oversimplification of the underlying complexities involved, however potential of applicability remains. Moreover, it is very likely that digestive processes will further influence the starch digestibility and subsequent mechanistic interpretation *in vivo*, so human trials investigating postprandial blood glucose levels after consumption of these breads would be extremely informative.

4. Conclusion

In previous studies, *Plantago* fibres extracted from different species differed in their physicochemical properties and subsequently their rheological behaviour and influence on digestibility. However, the simple nature of starch-fibre gel models used in those studies limit direct industry transferability. In this work, the impact of a 1% addition of *P. ovata* fibre and *P. turrifera* fibre was explored in a formulated starch-based food product, rice-based gluten-free bread. To compensate for high water absorption capacity of the fibres, water level was adjusted to produce doughs with equal consistency according to empirical dough measurements. Dough with the *P. ovata* fibre had the highest firmness, cohesiveness, and consistency. Specific volume and texture properties were also superior in bread with *P. ovata*. Both fibres had positive effect on storage stability, as

their addition resulted in lower moisture loss and lower change in hardness than in the rice flour control bread. Starch hydrolysis revealed that *P. ovata* fibre did not change digestibility behavior, while *P. turrifera* increased hydrolysis rate, but decreased hydrolysis extent. Comparing these trends with results regarding simple rice starch-fibre composite gels, we see a certain level of transferability. For future directions, we recommend rheological and leavening studies of the dough and microscopy of the bread particularly with varied addition levels, could shed further light on the hypothesized mechanisms. Moreover, developing a gluten-free recipe with increased protein addition and *Plantago* fibre would be an interesting and useful way of improving nutritional value of gluten-free bread.

5. Literature

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CHAPTER 8

Conclusions and future directions

Conclusions

Work presented in this thesis focused on exploiting fractionated fibre of *Plantago ovata* (psyllium) and underexplored *Plantago* species as modulators of quality in starch gels and starch-based products.

Firstly, a comprehensive literature review rationalised the application potential of said fractions for practical use (Chapter 2). We addressed benefits and limitations of psyllium husk implementation in food products, and proposed solutions with high-level rationale. To assess suitability of alternative *Plantago* species, we analysed their monosaccharide composition, FTIR spectra, and in-depth rheological properties, which showed remarkable resilience under deformation of *Plantago turrifera* and *Plantago drummondii* (Chapter 3). This enables us to recognise their suitability as stabiliser and texturisers for food product development as valuable option besides *Plantago ovata*. Correlation analysis illustrated potential structure-function relationships, aiding us in understanding the underlying viscoelastic properties. In Chapter 4, addition of these fibre fractions to rice starch showed significantly decreased freeze-thaw syneresis and changes to their viscoelastic profile. Most of fibres significantly increased viscoelastic moduli of composite rice starch-fibre gels, decreased their yield point, and significantly increased their flow point, again, confirming their potential as improvers of food structure. However, all fractions except one increased hydrolysis rate. We attributed this to tri-phasic structures where the interstitial water allows for locally increased enzyme concentrations. Our next experiments focused on a specific fraction, extracted with 65 °C water from *P. ovata* seeds (POM), which showed decreased starch hydrolysis rates in Chapter 5. In this work, we chose five different starches based on their amylose content, and fabricated composite gels, observing them at three different storage conditions. Microscopy, syneresis behaviour, and rheology enabled us to form hypotheses on their mechanisms and interactions. We showed synergistic interactions between amylose and POM, and antagonistic relationship between amylopectin and POM. Overall, the viscoelastic properties of composite gels are driven by the structure of gelatinised starch and structural properties of the added fibre. Chapter 6 presented a simplified fractionation method for psyllium husk, exploring more commercially-relevant processing of fractions. By combining two fractions with three corn starches with varying amylose content at three different temperature treatments, we gained an understanding of their structural and functional properties. One fraction increased

gelatinization and starch digestibility, while the other fraction significantly changed pasting profile and had a positive effect on starch hydrolysis. We hypothesised that this behaviour was due to the markedly different water absorption behaviours and relatively temperature-independent viscosification in one fraction and temperature-dependent changes in gelling in the other. A key highlight of this chapter also provides important consideration for food industry use, which is that simple processing can yield significant differences in the final product. Finally, in Chapter 7, we applied fibre fractions from *P. ovata* and *P. turrifera* (identified in Chapters 3 and 4 to have differing chemistry and rheological behaviour) to rice gluten-free bread to examine their impacts in a complex food system. In this study hydration level was adjusted according to each bread recipe. Both fibres improved storage stability, but *P. ovata* had overall improved bread quality. Moreover, their impact on starch hydrolysis also differed – *P. ovata* did change hydrolysis behaviour, while *P. turrifera* potentially increased resistant starch, but also showed an increase in hydrolysis rate.

Overall, this thesis presents possible strategies to diversify the application of fibre from *Plantago* mucilage in food products, particularly in starch-based food products. The main insights from this work show that fractionated fibre from the same source has extremely variable rheology properties, ranging from viscoelastic fluid to strong gel. This is particularly important, as it is most often reported that covalently crosslinked gels can be described as strong gels, however here it is shown that physical gel (fraction I from *P. drummondii*, at 1% concentration) forms strong and resilient gel. Moreover, it is important to highlight that this thesis contributes to the advancement of knowledge and application of the mucilaginous fibres and their characterization by presenting the first ever rheological characterization of *P. turrifera* and *P. drummondii*. In addition to rheology, starch hydrolysis by α -amylase showed interesting trends (increase in the hydrolysis parameters) whose mechanisms should still be fully explored. Nevertheless, these results indicate that comparison between different methods of starch hydrolysis should be utilised to fully understand the effects of fibre on the starch digestion. The in-depth study of interactions between starch and *P. ovata* fibre confirmed the role of amylose and amylopectin as being the key drivers of gel structure which, again, was not explored in previously published research. Next, the viscosity behaviour of psyllium-starch gels at high temperature (>95 °C) was presented for the first time and confirmed the relevance of chosen fibre fractions as well as the amylose to amylopectin ratio

on the structure and function of fibre-starch gels. This thesis brought new insights into characterisation and utilisation of *Plantago* fibre, as well as opportunities for future research and development, which are presented below.

Limitations and future directions

1. Structural characterization of *Plantago* fibre

As mentioned in Chapter 3, we recognise that molecular weight data is most likely not entirely accurate, due to experimental and instrumental limitations. We also recognise that molecular weight and intrinsic viscosity are important polysaccharide attributes that are highly related to their function, and therefore, we plan to further develop appropriate processes for molecular weight determination of *Plantago* fibre by using ionic liquid based size exclusion chromatography which will be done in collaboration with University of Leeds. Moreover, further structural characterization by linkage analysis, NMR (nuclear magnetic resonance), or PACE (Polysaccharide Analysis using Carbohydrate gel Electrophoresis) would provide new insights which would also complement monosaccharide analysis and rheological studies.

2. Determination of starch hydrolysis

While the described method provide an important understanding of impact of fibre on starch hydrolysis, the methodological constraints which did not account for viscosity increase caused by fibre necessitate cautious interpretation and suggest avenues for further inquiry, such as use of other starch digestibility protocols, such as INFOGEST*. Moreover, *in vivo* experiments on starch digestibility would be extremely useful.

* Brodkorb, A., Egger, L., Alminger, M. *et al.* (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat Protoc* **14**, 991–1014.

<https://doi.org/10.1038/s41596-018-0119-1>

3. Further rheological studies

Temperature sweep measurement would be important addition to profiling *Plantago* fibre and starch-fibre gels, and we aim to carry out these experiments soon.

Temperature sweep fibre profile would also be useful for understanding viscoelastic behaviour under high temperature, which is often applied in food industry.

Another rheology experiment would be viscosity measurement of starch digestibility mixtures, particularly in a method where samples vary in viscosity (such as suggested above).

4. Expanded fibre concentrations range

While this study provided valuable insights into fibre impact on starch gels, the limited concentration range suggests a need for larger studies to 1) confirm current findings, 2) help expand on mechanistic theories, 3) examine applicability of higher concentration in food products and food manufacturing.

5. Using size exclusion chromatography for leached starch polymers

As stated throughout this thesis, one of our main goals was to understand interactions between starch and *Plantago* fibre. Leaching of starch polymers during hydrothermal treatment is affected by added fibre, therefore a SEC profile of leached polymers (with and without presence of fibre during thermal treatment) would enable us to understand if fibre interacts with polymers of a particular molecular weight, and if it suppresses or promotes leaching of specific starch polymers.

This thesis highlights promising opportunities for both research and commercialization. Given that the materials explored in this work are fibres, their incorporation into food products offers significant potential for the development of healthier dietary options. The rising incidence of colon cancer, which is inversely correlated with fibre intake, is a growing concern across all age groups, particularly among young adults. As such, increasing fibre consumption should be a key dietary goal, a recommendation consistently emphasized in medical and health-related initiatives. Developing fibre-rich food products presents a valuable approach to helping individuals meet their daily fibre requirements.

In-depth exploration of three possible strategies to diversify the application of fibre from *Plantago* mucilage in food was presented and explored: natural variation, fractionation, and combined.

The use of fibre from *Plantago* species, as an alternative to *P. ovata*, emerges as a promising avenue for future research, particularly in enhancing rheological properties. These fibres present a potential ingredient for structuring and achieving products with specific functional characteristics. Among the species, *P. drumondii* has demonstrated notable resilience, making it suitable for products that require high-temperature processing or extensive handling, such

as doughs (e.g., pizza dough, pastries), extruded products, meat products (e.g., sausages), and confectionery (e.g., caramels). Due to its robustness, *P. drumondii* fibre ensures that the product maintains its structural integrity and texture during manufacturing. In comparison, *P. turrifera*, while also resilient, exhibits more viscous properties, making it ideal for products that undergo significant handling but require a softer texture. Examples include dairy products (such as yogurt, cream cheese, and dairy spreads), jams, jellies, frozen foods, and pourable foods (e.g., soups, stews, sauces).

Both *P. drumondii* and *P. turrifera* fibres are particularly well-suited for the development of dysphagia-friendly foods. This is of increasing importance as the global population ages, leading to a higher demand for accessible and safe food options for individuals with dysphagia.

The process of fractionation plays a critical role in this work, providing valuable insights into the rheological properties of different fibre fractions from the same species. These fractions exhibit distinct rheological profiles, offering a range of textures suitable for various food products. Additionally, fractionation offers a practical solution to a challenge faced by food manufacturers using psyllium. The annual production and supply of psyllium can be volatile, heavily influenced by climate and rainfall, leading to inconsistent quality and potential losses for manufacturers. Typically, psyllium is sold and used in its husk form, and, to date, no manufacturer has explored fractionated psyllium fibre. Fractionation, however, could address this issue by removing the fraction extractable in cold water, allowing the remaining fibre to retain its gelling and texturizing properties. Scaling up the fractionation process to meet the needs of individual companies is a feasible next step.

While the fractionation process conducted in this study used relatively small volumes (up to 800 mL), several insights have been gained that could facilitate scalability. First, processes involving alkaline or acidic solvents are likely to be time and resource-intensive, as neutralization would be required. Furthermore, given that fibre concentration cannot be precisely determined without drying, batch-to-batch adjustments would be necessary. Therefore, a water-based extraction process, which is food-safe and can yield gels with varying structural capabilities depending on the extraction temperature, is recommended. Second, the centrifugation or filtration of extracted fibre, to separate it from the seed or husk, will need to be optimized. This presents an industrial challenge, as *Plantago* fibre gel sets rapidly, making separation difficult even at small volumes. One potential solution could be to perform

the separation at the temperature at which extraction occurred, as the fibre would still be soluble. Additionally, high-speed, high-power centrifuges may be considered, but manufacturers must evaluate whether the use of such equipment or maintaining extraction temperatures would be the more cost-effective option. Finally, the simpler fractionation method presented in this work offers distinct advantages. The one-step process yields fibres with variable properties, which can be applied to specific products and purposes. Starting with a simpler method and refining it through additional steps, as needed, is advisable.

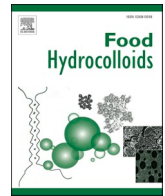
The findings from this research provide transferable knowledge for other highly viscous soluble fibres. Specifically, seeds such as linseed (flaxseed), chia, and basil—which are commercially available, widely used in recipes, and form gels upon contact with water—can also benefit from the fractionation process. Among these, linseeds absorb the least amount of water, creating a viscous fluid rather than a gel, making them less suited for fractionation. Additionally, linseeds produce less mucilage, resulting in lower fibre yields, which may not be economically viable. In contrast, chia, basil, and psyllium seeds can absorb higher amounts of water and form gels, making them more suitable for fractionation. Notably, while psyllium forms a stronger gel compared to chia and basil, this study demonstrates that Australian native *Plantago* species form even stronger gels, indicating that the fibres—and their fractions—from all these species have targeted applications in food products.

In conclusion, the work presented in this thesis highlights the significant potential of *Plantago* fibres in food products, offering the ability to achieve diverse structural, textural, and functional properties. It underscores the promise of underexplored, native Australian species as novel fibre ingredients. Several avenues for both commercial and research applications have been identified. Ultimately, these findings contribute to the advancement of healthier, more accessible food options with targeted functionalities.

Appendix I

Cowley, J. M., Ren, Y., Štrkalj, L., Foster, T. J., & Burton, R. A. (2025). The influence of mucilage-rich flours from diverse Australian *Plantago* species on the pasting, storage, and gluten-free breadmaking properties of rice flour. *Food Hydrocolloids*, 160, 110788.

This research paper shows changes in pasting, storage, and breadmaking of gluten-free rice bread when whole seed flour of several *Plantago* species is added. This also represents one of the solutions proposed in Chapter 2, literature review, where we suggested that the use of whole seed flour would be less wasteful for food industry.



The influence of mucilage-rich flours from diverse Australian *Plantago* species on the pasting, storage, and gluten-free breadmaking properties of rice flour

James M. Cowley^{a,*}, Yi Ren^b, Lucija Štrkalj^{a,b}, Tim J. Foster^b, Rachel A. Burton^a

^a School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA, Australia

^b Division of Food, Nutrition and Dietetics, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK

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ABSTRACT

Plantago seed composition varies with natural variation, particularly regarding their mucilage content and structure, but little is known about how this influences their potential as replacements for conventional food hydrocolloids. We investigated the influence that whole seed flour (WSF) addition from diverse *Plantago* species had on the pasting, storage and gluten-free (GF) breadmaking properties of rice flour (RF). Despite being starchless, *Plantago* WSF significantly altered the pasting profiles of RF with the mucilage conclusively shown to be key. Hastened pasting, stimulation of gelation on cooling and increased storage stability were hypothesised to be related to starch-hydrocolloid interactions. GF RF doughs with WSF added had significantly increased elastic moduli (G'), with $\tan \delta$ showing a reduced frequency dependence. Improved dough rheology modulated proofing kinetics and led to collapse resistance which produced GF breads with increased specific volume, improved crumb structure, and improved textural characteristics like lower hardness and higher cohesiveness. WSF from the commercial species, *P. ovata* (psyllium) produced high quality breads but two Australian native relatives, *P. turrifera* and *P. cunninghamii*, produced doughs with improved rheological properties and GF breads with greater volume, improved crumb structure and improved texture. Effects of *Plantago* WSF addition were partly independent of mucilage content suggesting that mucilage polysaccharide structure is influential and might be related to co-polymer network formation. These data show that *Plantago* WSF can be used as nutritious and sustainable hydrocolloids in starch-based foods and that natural variation in *Plantago* seed composition is an untapped resource for selectively manipulating their quality.

1. Introduction

Coeliac disease is a chronic autoimmune enteropathy triggered by ingestion of gluten proteins from wheat, barley and rye. Gluten-related immune responses in the digestive system trigger gastrointestinal irritation and inflammatory lesions, eventually leading to villi atrophy and associated malabsorption conditions (Green & Jabri, 2003). Currently the only effective treatment for the 1%–2% of the population that suffer from coeliac disease and other gluten-related disorders is adherence to a strict, lifelong gluten-free (GF) diet (Anton & Artfield, 2008). In those following a GF diet, there is often a demand for GF versions of typically gluten-containing products like baked goods which must be enhanced with a suitable texture modifier to have consumer acceptance. As such, the market for GF products, and the texture modifiers required for their

production, is ever-growing (Roman et al., 2019).

Leavened breads are arguably one of the most difficult baked goods to create without gluten. In breadmaking, the entanglement of gluten proteins creates a mechanically-robust three-dimensional network capable of trapping CO₂ during leavening allowing a dough to rise effectively giving it an airy yet robust texture, essential to consumers (Haque et al., 1994; Shewry & Tatham, 1997; Tilley et al., 2001; Wieser, 2007). GF breads are unable to rise effectively and so have low volume, have a powdery or crumbly texture and high crumb hardness (Hager & Arendt, 2013). GF bread must therefore be augmented with ingredients and additives that can replicate the viscoelastic properties of gluten in order to produce products with satisfactory consumer appeal (Lazaridou et al., 2007). Non-gluten flours and purified starches are the most common base ingredient which is often combined with one or more

* Corresponding author.

E-mail address: james.cowley@adelaide.edu.au (J.M. Cowley).

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hydrocolloids to strengthen and viscosify the dough (Anton & Artfield, 2008). The most commonly-used polymer for gluten replacement is hydroxypropylmethylcellulose (HPMC), a semi-synthetic derivative of cellulose with excellent viscoelastic properties (Foschia et al., 2016). While HPMC is found in 40%–50% of GF breads, consumers tend to respond negatively to such synthetic hydrocolloids in the ingredient list (Horstmann et al., 2018; Montemurro et al., 2021), making natural polysaccharides, like those from plants, a more appealing alternative. Plant exudate gums like gum arabic are extruded from a point of stress (usually physical injury or fungal infection) and collected, and seed gums like guar or locust bean gum are chemically extracted from endosperm tissue (Izydorczyk et al., 2005; Pollard et al., 2008). These types of plant gums require processing and refinement, while another class of plant hydrocolloid, seed mucilage, simply expands from the wetted seed coat of some species in a process called myxospermy (Phan & Burton, 2018). The most commercially significant seed mucilage is psyllium gum, which is milled from the seeds of *Plantago ovata* and used extensively as a dietary fibre supplement and more recently as a functional food ingredient (Madgulker et al., 2014). Psyllium gum is predominantly a complex heteroxylan with a structure that has proven difficult to define, but the current data consensually define the polysaccharide as having a β -(1,4)-linked-D-xylopyranose backbone, variably substituted at O-2 and/or O-3 positions with mono-, di- and oligosaccharide substitutions of α -L-arabinofuranose and β -D-xylopyranose in various combinations and linkages (Fischer et al., 2004; Phan et al., 2016, 2020; Yu et al., 2017).

Psyllium gum is often used to replace gluten in GF baking. Early studies reported that the fibrillar ‘weak gel’ network of psyllium gum was sufficient to support gas cell formation and facilitate proofing of GF doughs (Haque et al., 1993, 1994; Haque & Morris, 1994). This was later supported in work by others (Cappa, Lucisano, & Mariotti, 2013; Fratelli et al., 2021; Fratelli, Muniz, Santos, & Capriles, 2018; Mancebo, San Miguel, Martínez, & Gómez, 2015; Ren, Linter, Linforth, & Foster, 2020c). Fundamentally, the addition of psyllium improves workability of GF dough (Mariotti et al., 2009), likely as a result of the improved rheological properties of the dough that have also been reported (Collar et al., 2015; Mancebo et al., 2015). Zandonadi et al. (2009) showed that improved dough and bread qualities led to good acceptability of psyllium-containing GF breads in both coeliac and non-coeliac individuals. The overwhelming majority of these studies have focussed on purified psyllium gum, the production of which is wasteful, as 75% of the seed is discarded after removal of the husk (containing the psyllium gum) (Cowley & Burton, 2021; Cowley et al., 2022). Whole psyllium seeds contain 7.5 times more protein, 3 times more lipids, less sodium, more minerals including zinc and magnesium, and beneficial compounds like acteoside and antioxidants that are not present in the husk (Patel et al., 2016, 2019; Ziemichód et al., 2018). *Plantago* seeds also contain low molecular weight saccharides and mannan (Cowley et al., 2021) which may have probiotic and/or nutritional properties (Ang et al., 2023; Jonathan et al., 2012; King et al., 2017), and while nutritional studies using whole psyllium seeds are rare, one study found that psyllium seed consumption reduced serum cholesterol (Gelissen et al., 1994). The use of a whole seed *Plantago* flour, which contains the functional mucilage polysaccharides along with other beneficial phytonutrients, is a way to reduce waste, bolster a product’s nutritional profile, while improving its functional properties.

Whole seed flours (WSF) of myxospermous seeds are rarely seen in the literature as hydrocolloid additions in foods, but they have recently gained interest due to their ease of processing, sustainability merits, and additional nutritional benefits normally lost during hydrocolloid extraction. Flaxseed (*Linum usitatissimum*) and chia (*Salvia hispanica*) WSF are the most reported. The incorporation of flaxseed WSF has repeatedly been reported to increase specific volume and reduce hardness of GF baked goods (Kumar, Bejkar, Du, & Serventi, 2018; Ozkoc & Seyhun, 2015; Pruska-Kędzior et al., 2008; Sung & Chai, 2017; Ziemichód, Różyło, & Dzik, 2020; Ziemichód, Wójcik, & Różyło, 2018),

while chia WSF improves the mixing and thermal properties of GF flour (Moreira et al., 2013) subsequently improving quality and acceptability of GF breads (Sandri et al., 2017; Steffolani et al., 2014). Studies incorporating *Plantago* WSF are even rarer. Pejcz et al. (2018) found that adding *Plantago ovata* (blond psyllium) and *P. psyllium* (dark psyllium) WSF to wheat bread improved dough hydration and improved the antioxidant content but competition with gluten for water was observed. This was corroborated by Hadidy (2020) who supplemented wheat-based pan breads and saw an increase in farinographic water absorption and a reduction in dough extensibility. Ziemichód et al. (2018) reported that the addition of whole and ground *P. psyllium* seeds increased the specific volume of GF bread while *P. ovata* whole and ground seeds had no significant effect. Pejcz and Burešová (2022) found that these additives reduced specific volume but did positively influence texture of GF bread. These and all known studies using *Plantago* seed components as ingredients in baking, including purified gums, have been confined to *P. ovata* and limitedly, *P. psyllium*, opening the opportunity to study the suitability of the 200+ other members of the *Plantago* genus (Elliot & Jones, 1980). This is especially interesting because of reports of significant diversity in the composition of seed mucilage of Australian native *Plantago* species possibly related to structural complexities like heteroxylan branching patterns (Cowley et al., 2021; Phan et al., 2016). Other researchers have reported that for *Plantago ovata*, rheological and functional differences in fractionated seed mucilage were a result of varied heteroxylan branching patterns (Marlett & Fischer, 2003; Ren, Linter, Linforth, & Foster, 2020c; Yu et al., 2017, 2019; Yu, Yakubov, Martínez-Sanz, Gilbert, & Stokes, 2018; Zhou et al., 2020). Our recent work showing significant variation in the content, fractionation profile, water absorption capacity and chemical profile of seed mucilage among diverse *Plantago* species from Australia highlighted their potential as sources of hydrocolloids (Cowley et al., 2021). Importantly, consistent trends in the mucilage chemistry especially increasing ratios of arabinose to xylose with increased extraction intensity (indicative of increased substitution levels) were shared among the species, allowing extrapolation from detailed previous studies on *P. ovata*. Adding to this, our finding that WSF from different *Plantago* species contain high levels of protein, omega-3 fatty acids, and fermentable dietary fibres that are/would be lost in current hydrocolloid processing, further highlights the potential for using WSF in particular as highly-nutritious hydrocolloid replacements in food technology.

We hypothesised that variation in mucilage properties among seeds of diverse *Plantago* species would result in differences in in-food functionality that could be exploited to improve product quality. In this work, we studied the impact of the addition of whole seed flour (WSF) from commercial psyllium, *Plantago ovata*, and six of its relatives found in Australia (three naturalised, *P. coronopus*, *P. lanceolata* and *P. major*, and three Australian native, *P. cunninghamii*, *P. turrifera* and *P. debilis*) on the pasting, storage and GF breadmaking properties of rice flour.

2. Methods and materials

2.1. Materials

Rice flour was purchased from Doves Farms (Hungerford, United Kingdom) and was determined to have a moisture, protein, ash, lipid and amylose content of 11.1%, 7.23%, 0.42%, 2.8%, and 28.8%, respectively, based on previous work using the same flour batch (Ren et al., 2020a). Bulk seed was harvested from *Plantago* species that were grown to maturity as per Cowley et al. (2021). Original seed stocks of *P. ovata* and *P. major* seeds were purchased commercially while seeds of *P. coronopus* (naturalised), *P. lanceolata* (naturalised), *P. cunninghamii* (native), *P. turrifera* (native), and *P. debilis* (native) were wild collected in Australia in compliance with relevant institutional, national, and international guidelines and legislation. An eighth species with seed composition characterised to the same depth as other species used here, *P. bellidioides* (native), was included in pasting experiments but not

subsequent experiments used for all other species due to material limitations. Seed was ground to flour using a MM400 Mixer Mill (Retsch, Haan, Germany) and graded to 0.5 mm. Additionally, mucilage-depleted flour from *P. ovata* was produced by extracting mucilage in hot water (80 °C) for 1.5 h under constant stirring (Phan et al., 2016) before decanting liberated mucilage, freeze-drying seeds to a constant weight, and milling as described above. This method is sufficient to remove the majority of the mucilage, but some remains, hence we refer to this as mucilage-depleted. Mucilage depletion (compared to a mock soaked at room temperature for the same length of time without agitation) was confirmed visually by staining with 0.01% ruthenium red as per Cowley et al. (2022).

Caster sugar, table salt and sunflower oil were purchased from Sainsbury's (Holborn, United Kingdom) and yeast (*Saccharomyces cerevisiae*) was purchased from Allinson (Maidenhead, United Kingdom). All materials were stored dry at room temperature except for the yeast which was stored dry at +4 °C.

For these experiments, a 4% addition of *Plantago* WSF was selected as based on previous work using a 1% psyllium husk addition satisfactorily replaced gluten in a model GF bread based on the same rice flour (Ren et al., 2020c). As *P. ovata* WSF contains c. 25% psyllium husk (Cowley & Burton, 2021), 4% WSF addition was considered to correspond approximately to a 1% psyllium husk addition and produced satisfactory breads in pilot experiments.

2.2. Measurement of pasting properties

To create suspensions, 100 mg (4% addition) of *Plantago* flour was blended with 2.5 g of rice flour directly in the RVA cannisters. Twenty-four grams of deionised water was added to the mixture and mixed briefly by vigorous stirring to disperse any clumps. Control samples lacking *Plantago* flour were prepared following the same protocol. Additionally, a pre-dispersed suspension of WSF was also prepared by adding 100 mg to 24 g of deionised water in a beaker, which was stirred with a magnetic flea for 30 min at room temperature before transferring to an RVA cannister and continuing the protocol as described below.

Pasting properties of rice flour-*Plantago* flour blends were determined by Rapid Visco Analysis using an RVA Super 4 (Newport Scientific, Warriewood, Australia). Suspensions were held at 25 °C for 2 min before heating to 95 °C at 12 °C/min, holding at 95 °C for 2.5 min before cooling to 25 °C at 12 °C/min where they were held for a final 2 min. For the first minute, the suspension was mixed at 960 RPM. The remainder of the test was performed under constant stirring at 160 RPM.

The pasting parameters extracted were: pasting temperature (the temperature at which viscosity rapidly increases as a result of starch granule swelling upon the uptake of water), peak viscosity (the maximum viscosity obtained during heating), time to peak viscosity, trough viscosity (the minimum viscosity obtained during hold at maximum temperature), breakdown viscosity (difference between peak and trough viscosities), final viscosity (viscosity at completion of the run), and setback viscosity (difference between final and trough viscosities).

Pasting properties were measured in triplicate.

2.3. Dough properties

2.3.1. Dough rheological properties

Viscoelastic properties of dough at baking formulations levels (Table 1; excluding yeast) were determined by dynamic oscillatory tests on a Physica MCR 301 Rheometer (Anton Paar, Graz, Austria) using 25 mm serrated parallel plate geometry (PP25/P2) with a gap of 2 mm. Excess dough was trimmed and the newly exposed surface was coated in a thin layer of mineral oil to prevent moisture loss. Samples were rested for 500 s. Oscillatory measurement of the storage (G') and loss (G'') dynamic moduli was performed at 30 °C (proofing temperature) within a frequency range of 0.6–600 rad/s. The frequency sweep was performed

Table 1

Gluten free bread formulation.

Ingredient	Amount (per 100g of rice flour)
Rice flour	100 g
Sugar	5 g
Salt	2 g
Yeast	1.5 g
<i>Plantago</i> flour	4 g
Water	120 g
Oil	5 g

at a constant strain of 0.02%, within the linear viscoelastic region which was determined by amplitude sweep (0.01–10 000% strain) previously (data not shown). The dynamic moduli, G' and G'' , and the loss tangent ($\tan \delta$), calculated as the ratio between G'' and G' , were extracted at two frequencies deemed representative of low (0.129 rad/s) and middle (6 rad/s) frequency ranges.

2.3.2. Dough proofing dynamics

Proofing kinetics were studied following Vidaurre-Ruiz et al. (2019) with modifications. Doughs prepared at baking formulation levels (Table 1) were moulded into a cylinder (Ø 22 mm) in the centre of a 90 mm Petri dish. Cylinders were removed and the doughs were proofed in an incubator at 30 °C for 85 min. Dishes were scanned (C7270i, Canon, Japan) prior to and after rest and every 10 min during proofing. Images were analysed using the measurement function of Photoshop CC 2018 (Adobe, San Jose, USA). Rest spread (RS) was calculated as the difference in dough circumference at 0 min and 10 min. Proofing changes are presented as the difference between the dough circumference at the time of measurement and after 10 min rest.

2.4. Breadmaking

The impact of additions of *Plantago* flour to baking quality of a rice flour-based gluten-free bread was assessed with baking tests. The effect of *Plantago* flour alone was studied by a 4% addition to a rice flour-based formulation (Table 1) based on work by Ren et al. (2020a) without other hydrocolloids or purified starches. Control breads were produced without the addition of *Plantago* flour.

Doughs and breads were prepared at between two and four replicates (based on material limitations) and baking and subsequent analyses were performed within one day.

2.4.1. Baking procedure

Water and oil were added to the combined dry ingredients and mixed in a multi-speed tilt-head stand mixer (Chef Premier, Kenwood Ltd, Havant, United Kingdom) with a silicone-edged creaming beater (AT501, Kenwood Ltd, Havant, United Kingdom) for 7 min at a constant speed. 200 g of dough was transferred to a baking paper-lined multi-size (7.5 cm × 7.5 cm × 10 cm) anodised cake pan (10034, Silverwood, Birmingham, United Kingdom). The pan was sealed with cling film to maintain humidity and doughs proofed at 30 °C for 85 min in an incubator (KB115, Binder, Tuttlingen, Germany). Proofing of each dough formulation was monitored at the time of baking tests by placing a 10 g sample of dough into a measuring cylinder and proofed at 30 °C for 85 min, recording the volume every 10 min. The change in dough volume is presented as the difference between the volume at the measurement time and the initial volume.

Loaves were baked at 230 °C for 40 min in a deck oven (Compacta, Tom Chandley, Hyde, United Kingdom), then cooled to room temperature for 1 h before measurements were taken.

2.4.2. Bread quality tests

Bake loss (water and CO₂ released during baking) was recorded as the difference between dough weight and loaf weight (Equation (1)).

$$\text{Bake Loss (\%)} = \frac{\text{Weight of dough} - \text{weight of loaf}}{\text{Weight of dough}} \times 100 \quad \text{Equation 1}$$

Loaf volume was measured as the displacement of a mass of rapeseeds of known density. Specific volume was calculated as the ratio of the loaf volume to the loaf weight.

For subsequent quality tests, each loaf was sliced into 1.25 cm thick slices (four full slices, two crusts) using an electric knife and bread slicing guide.

Loaf crumb structure including alveolation and slice structure was evaluated on the two inner slice surfaces using the C-Cell Bread Imaging System (Calibre Control International Ltd., Warrington, United Kingdom) as per the manufacturer's standardised procedure.

Crumb colour of the central slice surfaces were measured by colourimeter (ColorQuest XE, HunterLab, Reston, United States of America) as per the manufacturer's standard procedure. To account for variation in crumb brightness caused by bread porosity (Ayustaningwarno et al., 2021), five measurements were taken at random across the subsample and averaged for a single replicate. Values L^* , a^* , and b^* were obtained and the difference values between control and sample loaves (ΔL^* , Δa^* , and Δb^*) and the total colour difference ΔE^* (CIE 2000) were calculated using the ColorTools add-in for MS Excel (<http://rgbcmky.com.ar/en/xla-2/>). Hue Angle was calculated using Equation (2) (Fernandes & Salas-Mellado, 2017):

$$\text{Hue Angle (}^\circ\text{)} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad \text{Equation 2}$$

The hue family was categorised based on the hue angle: $15 \pm 7.5^\circ$ = warm red; $30 \pm 7.5^\circ$ = orange; $45 \pm 7.5^\circ$ = warm yellow; $60 \pm 7.5^\circ$ = mid yellow; $75 \pm 7.5^\circ$ = cool yellow.

A 30 mm cylindrical punch was used to remove a central representative subsample from each of the four slices for texture analysis. Crumb texture was assessed by texture profile analysis (TPA) using a texture analyser (TA.HDplus, Stable Micro Systems, Godalming, UK) fitted with a 30 kg load cell and 100 mm aluminium compression platen (P/100, Stable Micro Systems). Pre-test, test, and post-test speeds were 1 mm/s with a target of 65% strain. Hardness, springiness, chewiness, resilience and cohesiveness values were calculated by the standard TPA analytical macro.

Moisture analysis was performed by weighing the subsamples used for TPA in aluminium pans before drying at 105°C for 24 h and weighing again. Moisture content was calculated as the difference between fresh weight and dry weight.

Technical replicates run in each analysis of bread quality were averaged for each batch replicate prepared.

2.5. Statistical analysis

Data were analysed using GenStat 15th Edition (VSNi, Hemel Hempstead, UK). Statistical differences, where indicated, were analysed by one-way ANOVA using Tukey's HSD post-hoc test ($p < 0.05$). The kinetic parameters of syneresis and proofing were estimated by non-linear model in GenStat 15th Edition (VSNi, UK). Area under the curve (AUC) in Fig. 1 was calculated using Prism 9 (GraphPa, La Jolla, USA). Correlation matrix in Fig. 6 was generated using R studio (R Core Team, 2012) and the *corrplot* function (Wei & Simko, 2021). To perform correlations with *Plantago* seed characteristics (Cowley et al., 2021), pairwise complete observations within the *corrplot* function were used so that controls (containing no *Plantago* seed and thus without seed characteristics to correlate to) were excluded from the correlation.

3. Results and discussion

3.1. Differences in mucilage properties drive how *Plantago* whole seed flours alter the pasting behaviour of rice flour in a binary mixture

Hydrocolloids are well-known to alter the cooking properties of starches and flours due to their unique functional properties (BeMiller, 2011), improving the quality of starchy products by creating structure and controlling water mobility (Liu et al., 2019). Furthermore, hydrocolloid-related changes to the pasting properties of starch/flour may be demonstrative of some functional properties of the hydrocolloid itself. To preliminarily study how the presence of mucilage, a hydrocolloid abundant in *Plantago* whole seed flour (WSF), affects the cooking of a starch-based food system, we prepared two types of flour from *P. ovata* seeds: one with mucilage intact and one with >90% of mucilage removed by a crude hot water extraction (mucilage-depleted; MD) (Phan et al., 2016). When stained with ruthenium red, hydrated intact seeds showed a distinct mucilage envelope surrounding the seeds that prevented them from coalescing, while MD seeds had a drastically reduced mucilage envelope and so clumped together strongly (Fig. 1A). A robust mucilage envelope surrounding the seed is also clear on the unstained seed while MD seeds showed only a thin coating remaining (Fig. 1A). When the intact whole seed and mucilage-depleted seeds were milled to flour (WSF and MD WSF, respectively), they exhibited distinct effects on the cooking of rice flour (RF) studied by Rapid Visco Analyser (RVA) (Fig. 1B). A pre-dispersed suspension of *Plantago* flour at the equivalent of a 4% addition level (relative to RF) and in the absence of RF showed negligible changes in viscosity while MD WSF addition to RF also caused negligible changes to the pasting profile (Fig. 1B), with no significant difference in the area under the curve of the RVA profile (Fig. 1C). With the addition of WSF from *P. ovata*, the pasting profile was substantially

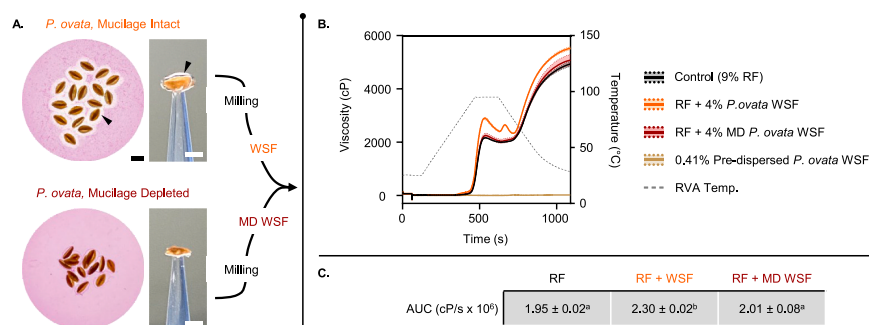


Fig. 1. Changes in the pasting profile and pasting properties of rice flour (RF) upon the addition of *Plantago* flour. **A.** Visual representation of mucilage on *P. ovata* seeds before and after mucilage extraction/depletion and milling to flour. Seed are stained with ruthenium red solution and unstained on a pipette tip to show the presence and depletion of the mucilage envelope (arrowhead). Scale = 2 mm. **B.** Pasting properties of rice flour in the presence of whole seed (WSF) and mucilage depleted (MD WSF) *P. ovata* flour demonstrating the key role of mucilage in pasting alterations. The profile of a 0.41% (equivalent to a 4% addition) *P. ovata* suspension is also shown. **C.** Bulk differences in pasting profiles are summarised by comparing the area under the curve (AUC).

altered, most notably with an increase in the maximum viscosities reached at both maximum and minimum temperatures (95 and 25 °C, respectively). Ultimately the bulk differences in RVA profile could be summarised crudely by area under the curve which was significantly higher than RF and RF with MD WSF (Fig. 1C), confirming that that mucilage polysaccharides are indeed acting as hydrocolloids and are the most impactful component of WSF. The remaining WSF material could be considered inert filler as it is mostly particles of insoluble fibre, protein and fat (Cowley et al., 2021). Besides the mucilage polysaccharides, we considered that native starch in *Plantago* seeds would also have a strong influence on the pasting properties and subsequent experiments. Whole seed monosaccharide profiles performed previously on these species did not specifically quantify the presence of starch, though glucose content of the whole seed was very low (>5% w/w) with at least part of this small quantity attributed to glucose-containing low molecular weight sugars (Cowley et al., 2021). As the enzymatic quantification of starch in *Plantago* seeds was inconclusive (probably due to the presence of mucilage; data not shown), we assessed the qualitative abundance of native starch in the *Plantago* seeds studied here using Lugol's iodine solution (Supplementary Fig. 1). Compared with barley (a starch-rich cereal), minimal to no positive starch staining was observed in *Plantago* seeds. No positive staining occurred in the endosperm or embryo tissues as would be expected of a starchy seed and only very minimal staining at the hilum was observed in four of eight species. This finding is in agreement with the pre-dispersed suspension of *Plantago* flour showing little viscosity and a mucilage-depleted *Plantago* flour contributing negligibly to the pasting profile of RF (Fig. 1B).

When comparing blends of RF with different *Plantago* WSFs that we

have previously shown to differ most significantly in their mucilage properties (Cowley et al., 2021), we observed correspondingly diverse impacts on all aspects of the pasting process (Fig. 2A and Supplementary Fig. 2) and extracted pasting properties (Fig. 2B). Prior to heating, negligible viscosity was detected regardless of *Plantago* type added (Fig. 2A). For the RF control samples (no *Plantago* WSF addition), viscosity began to rapidly increase at 88.95 °C (pasting temperature, PT; Fig. 2B). PT can be advanced or retarded in various ways by the presence of hydrocolloids (Shi & BeMiller, 2002). The influence of *Plantago* mucilage polysaccharides or mucilage-containing flours on the pasting/cooking of starches has not been widely examined, and where studied, has been limited to *P. ovata*. Cappa et al. (2013) studied the influence of blends of psyllium husk and sugar beet fibres on the pasting of a commercial gluten-free bread mixture and found that blends with a larger psyllium husk proportion (2.5% > 1.5% > 0%) reduced the pasting temperature but the effect was slight (~4 °C). Mancebo et al. (2015) reported a similarly small change when studying the effect of psyllium and hydroxypropylmethylcellulose on RF-based gluten free bread and dough qualities as did Belorio et al. (2020) when studying incremental substitution of maize starch with psyllium husk. We corroborate these findings by showing that *P. ovata* WSF (along with *P. coronopus* and *P. major*) had only a modest influence on PT of RF (0.4–4 °C reduction; Fig. 2B). This finding makes the strong reduction in PT by *P. cunninghamii*, *P. turrifera*, *P. bellidioides* and *P. debilis* WSF particularly interesting (Fig. 2B). These four closely related species all decreased PT by > 12 °C, manifesting as a smaller, secondary peak or shoulder that preceded the primary pasting peak (Fig. 2A). Bean and Yamazaki (1978) found that carboxymethylcellulose caused an increase in first-stage

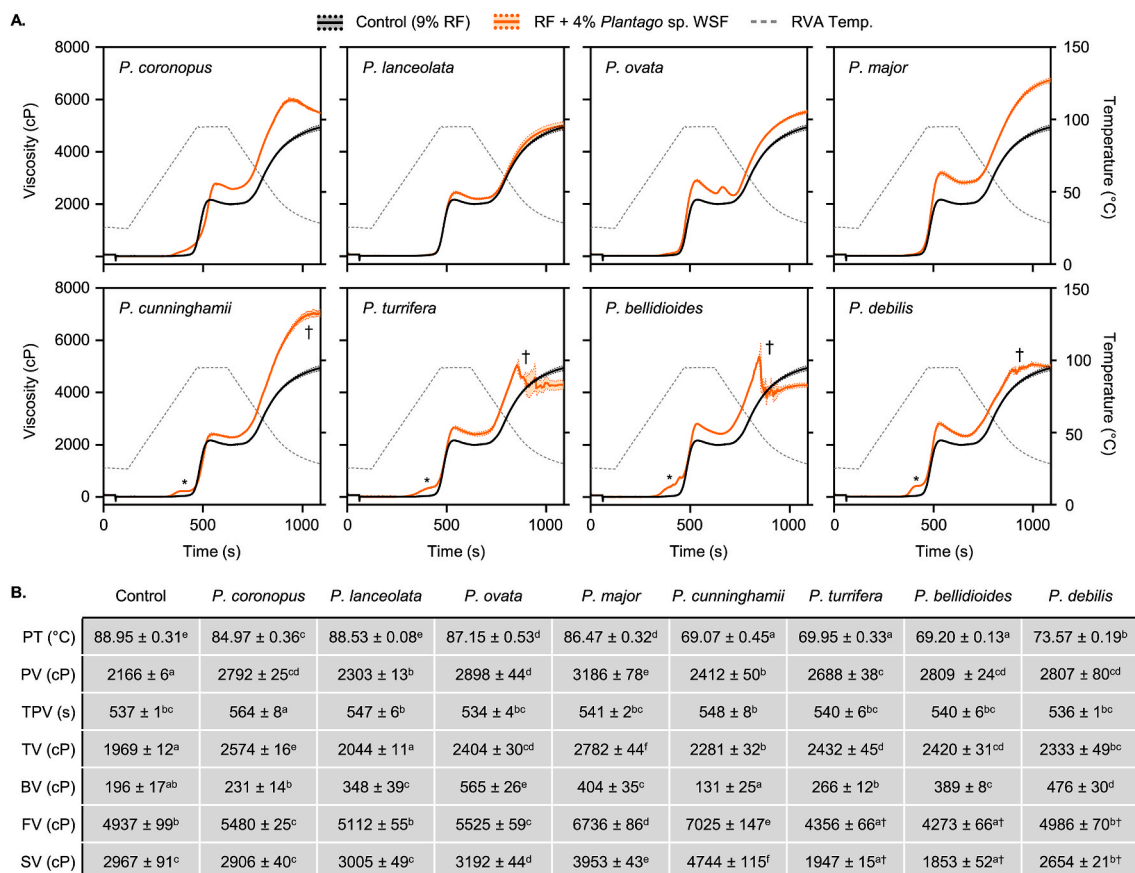


Fig. 2. A. Plots of pasting profiles of rice flour upon 4% addition of WSF from eight *Plantago* species compared to the control without *Plantago* flour. Solid lines represent the mean while dotted lines and area fill denote one standard deviation. First-stage granule swelling (if present) is marked with an asterisk while gelation-related noise is marked with a cross. For clarity, plots for each species are presented separately while the overlain version is presented in Supplementary Fig. 2. B. Extracted pasting properties. Values are means ± SD. Samples sharing the same superscript letter in a row are not significantly different ($p > 0.05$). Values that are inaccurate to the true viscosity due to gelation-related noise are marked with a cross.

granule swelling with Christianson et al. (1981) concluding that the initial stages of granule swelling were magnified in viscosified media (in their case, containing xanthan gum), as more work was required to move slightly swollen starch granules past one another. This effect is certainly possible as the excellent water holding abilities of the mucilage could viscosify the continuous phase and allow normally undetectable first stages of granule swelling to become exponentially magnified. However, seed water absorption capacity was not well correlated with pasting temperature nor the presence/absence of two-stage swelling (Fig. 6) suggesting that another mechanism is also at play. Some studies have suggested that many hydrocolloids can interact directly with small quantities of amylose leached during the initial stages of starch granule swelling, further enhancing and hastening the onset of pasting (Díaz-Calderón et al., 2018; Liu et al., 2003; Rong et al., 2022). It is possible that domains in mucilage polysaccharides shared by *P. cunninghamii*, *P. turrifera*, *P. bellidioides* and *P. debilis* allow these to interact directly with small amounts of amylose leached during the first stages of granule swelling.

Viscosification and co-polymer interaction are also likely to be involved in the effects on hot (peak viscosity; PV) and cold (final viscosity; FV) viscosity and their secondary pasting parameters, especially if granule swelling is altered. The addition of all *Plantago* WSFs but *P. lanceolata* significantly increased PV of RF (Fig. 2B). PV was increased most upon the addition of *P. major* WSF (47% increase) with more modest changes with other WSFs (11–33% increase). Importantly, time to peak viscosity (TPV) was unchanged in all samples except *P. coronopus* where its addition significantly delayed TPV by 27 s. The water holding capacity of the *Plantago* WSF polysaccharides likely increases PV by increasing the effective concentration of starch granules and promoting starch-starch interactions (Sullo & Foster, 2010) but it is likely that a composite effect is at play where both starch-starch and starch-hydrocolloid interactions contribute. It is known that interactions between hydrocolloids and granule-exuded amylose can hasten total amylose release and lead to a marked viscosity increases (Christianson et al., 1981; Freitas et al., 2003; Lai & Liao, 2002; Rojas et al., 1999). The stimulation of granule swelling seen in the reduced PT of RF might lead to more amylose being mobilised from the granule, free to form starch-starch and starch-hydrocolloid interactions, increasing the peak (PV) and subsequent viscosities (e.g. final viscosity; FV). *Plantago* species that had a larger share of mucilage fractions that were unextractable in cold water (hot water (HWE) and intense agitation extractable (IAE) (Cowley et al., 2021)), and more likely to form stronger physical gels based on previous rheological analyses (Yu et al., 2017), were more likely to lead to higher peak viscosities. Relatedly, while some pastes did not gel during cooling (i.e. *P. ovata* or *P. major*), Australian *Plantago* species (*P. cunninghamii*, *P. turrifera*, *P. bellidioides* and *P. debilis*) showed extensive evidence of gelation-related noise (Fig. 2A) where under the shear stress of the RVA paddle, a newly formed gel breaks and reforms continuously leading to significant variation in paddle torque that obscures the true viscosity (Lai et al., 2000). Such gelling during pasting has previously been seen when non-starch polysaccharides (mostly wheat arabinoxylan) were added to wheat starch (Sasaki et al., 2000) and high concentrations of psyllium husk were added to maize starch (Belorio et al., 2020). In those studies, it was concluded that the highly swollen, extensively leached starch granules occupied a greater volume in these samples and concentrated the non-starch polysaccharides and leached exudates leading to greater intermolecular interactions and gelation. Here, the extent and intensity of noise during retrogradation of RF, and thus the extent of gelation, is species dependent. Importantly, species with the greatest impacts on PT and largest peak shoulder related to first-stage granule swelling (*P. cunninghamii*, *P. turrifera*, *P. bellidioides*, and *P. debilis*) had the most extensive gelation suggesting that a related mechanism is involved. It is possible that amylose leached from swollen granules by interaction with Australian *Plantago* WSF polysaccharides (which we suggest is indicated by decreased PT and increased PV) and changes to granule disintegration (shown by large

breakdown viscosity values) lead to more molecules available to interact as the paste cooled (Weber et al., 2009). Such a phenomenon has been reported between extensively leached amylose and increasing concentrations of a complex polysaccharide from *Mesona chinensis* (Yuris et al., 2017). The polysaccharide was reported to interact with exuded but granule-bound amylose molecules and form a dense three-dimensional interlinked network between the polysaccharide, amylose and granules. In our previous work, these species varied in swelling and water absorption capacity but were similar in their mucilage yield and fractionation profile (Cowley et al., 2021). The four Australian native species that promoted PT, PV, and gelation upon cooling all had a larger share of fractions (>80%) that are likely to have gel-like behaviour (unextractable with cold water) as a result of high contents of arabinose and subsequent high ratio of arabinose to xylose (A:X) (Cowley et al., 2021; Yu et al., 2017). Previous work has shown that *Plantago* heteroxylans with higher A:X are more gel-like and might have helical structures/domains (Ren et al., 2020d; Yu et al., 2017). It is possible that the abundance of these types of heteroxylans in the presence of cooked starch can seed amylose aggregates in starchy matrices and initiate co-polymer network formation leading to synergistic increases in viscoelasticity seen here. Indeed, the presence of helical domains in *Plantago* with higher A:X levels might be necessary to allow heterotypic interactions with starch/amylose where high substitution density would typically prevent backbone-backbone interactions. In complex plant polysaccharides, blocky regions with regular substitution patterning can adopt a fold-dependent helix that exposes the backbone and allows network formation with other helical polymers like cellulose (Busse-Wicher et al., 2016; Grantham et al., 2017; Yu et al., 2018). This behaviour may include certain *P. ovata* heteroxylan fractions (Ren et al., 2020b) and might apply to amylose helices also. Such fold-dependent backbone-backbone interactions also have practical importance for hydrocolloids as they are thought to be critical to network formation in fenugreek gum which is an entirely substituted galactomannan (Cairns et al., 1987; Doyle et al., 2009; Goycoolea et al., 1995; Lundin & Hermansson, 1995; Morris et al., 1981). Most pasting parameters, however, were not explained well by the seed-relative fraction yield or mucilage-relative fraction proportion (Fig. 6) and thus we anticipate future work scrutinising motifs and rheology to be particularly informative.

3.2. Cooking rice flour with mucilage-containing *Plantago* WSF improves storage stability, possibly through system structuring

It is possible that the presence of co-polymer networks after cooking rice flour in the presence of *Plantago* WSF might lead to improved storage stability. Using pastes generated from RVA experiments, we measured syneresis over a 14-day storage period at +4 °C (Supplementary Fig. 3). By storage day 4, measurable quantities of water had been ejected through syneresis in the control, *P. coronopus*, *P. lanceolata*, and *P. ovata* pastes while remaining samples did not begin syneresis until day 6 (Supplementary Fig. 3A). Rates and magnitude of syneresis diverged substantially during storage where, by day 14 of storage, RF had lost substantially more mass through syneresis than samples containing *Plantago* WSF (Supplementary Fig. 3B). Samples containing *P. coronopus* and *P. lanceolata* reduced syneresis by ~25%, *P. ovata* by ~40% and the remaining samples an average of 50%. Syneresis was retarded the most in pastes containing *P. cunninghamii* WSF. High water-holding capacity will also reduce mass lost through syneresis (Lee et al., 2002) though the two were not significantly correlated (Fig. 6) indicating another or additional mechanism. The presence of a co-polymer network in samples could mean that the polysaccharides have the ability to 'outcompete' amylose resulting in fewer amylose-amylose interactions in favour of amylose-hydrocolloid interactions (Liu et al., 2003; Sudhakar et al., 1996). Fewer amylose-amylose interactions retard short-term retrogradation which in turn delays long-term retrogradation improving product stability. Such

an effect has been seen in starch gels containing xanthan gum (Dobosz et al., 2020). It is possible that the hypothesised interactions between starch and *Plantago* WSF polysaccharides seen in changes in the pasting experiments (Figs. 1 and 2), particularly in pastes containing the four Australian native species, are delaying the onset and reducing the magnitude of syneresis by creating phase continuity through a continuous network that resists the settling of particles and thus the migration of water to the surface of the gel. Indeed, *Plantago* flours with more gel-like characteristics (larger proportion of hot water- and intense agitation extractable mucilage fractions (HWE + IAE)/smaller proportion of cold water extractable mucilage (CWE)) retarded syneresis most (Fig. 6). However, like with other characteristics tested here, mucilage content was not significantly correlated with mass lost by syneresis (only fraction proportions) showing that its prevention by these WSFs cannot be explained simply by their mucilage content alone. Interestingly yield of liquid-like fractions (CWE) was positively correlated with mass lost by syneresis (Fig. 6) though this is likely a result of less robust mucilage envelopes overall in species with high CWE (i.e. CWE yield was strongly negatively correlated with proportion of HWE + IAE).

3.3. Rheological behaviour is enhanced in rice flour-based gluten-free doughs containing *Plantago* WSF, improving proofing behaviour

Based on the RVA and syneresis results, interactions of the mucilage polysaccharides with the starch matrix are likely, but the influence of rheological modification and viscosification of the whole system are also likely to be key to improving foods based on a starch matrix. We compared the influences that the addition of *Plantago* WSF to a more complex rice flour-based food matrix: gluten-free dough and bread.

The variation in rheological dynamic moduli of gluten-free doughs under frequency sweep are shown in Fig. 3A and B, while rheological parameters were extracted in the low and middle frequency range and are shown in Fig. 3C. In all formulations, the storage modulus (G' , elastic

behaviour) was greater than the loss modulus (G'' , viscous behaviour) regardless of frequency indicating solid-elastic behaviour (Fig. 3A), reflected in $\tan \delta$ (representing the ratio between viscous and elastic behaviour) which was always <1 (Fig. 3B). Rheologically, doughs containing *Plantago* WSF (excluding *P. lanceolata*) were more robust than control doughs as reflected in the significantly higher elastic moduli (G') across the frequency range. Reduced variability in the viscous modulus (G'') and subsequently lower variability in $\tan \delta$ across the frequency range further support that the *Plantago* WSF containing doughs have more typical solid-elastic behaviour, with the linear increase in G' with frequency showing that they behave particularly like soft gels (Wang et al., 2023). The addition of WSF of all species except *P. lanceolata* significantly increased dough elasticity (G') (Fig. 3C). These data correlate closely with visual appearance (data not shown) where control and *P. lanceolata* doughs were runny with a batter-like consistency while the remaining formulations were elastic and gel/dough-like, consistent with previous work using psyllium to replace gluten (Collar et al., 2015; Mariotti et al., 2009; Ren et al., 2021; Renoldi et al., 2021). Regardless of frequency, formulations with *P. turritifera* WSF dough had the greatest increase from the control dough. At the lower frequency, loss moduli extracted were similar between all formulations while at the middle frequency, larger differences are seen particularly from control and *P. lanceolata* doughs which were much lower in this range (Fig. 3C). This is also evident in the frequency sweep profiles, where control and *P. lanceolata* doughs showed higher frequency dependence than other doughs, particularly for G'' which was much higher at lower frequencies (<1 rad/s) (Fig. 3A). As a result, in control and *P. lanceolata* formulations, $\tan \delta$ was highly frequency dependent (due to the frequency dependence of G''), being high at low frequencies (<1 rad/s) and before steeply declining as frequency ramped until the middle frequency range (~ 6 rad/s) where it was lower than the other formulations (Fig. 3A and C). Other formulations were largely frequency independent in both G' and G'' and subsequently $\tan \delta$. The variability in viscous response/ $\tan \delta$

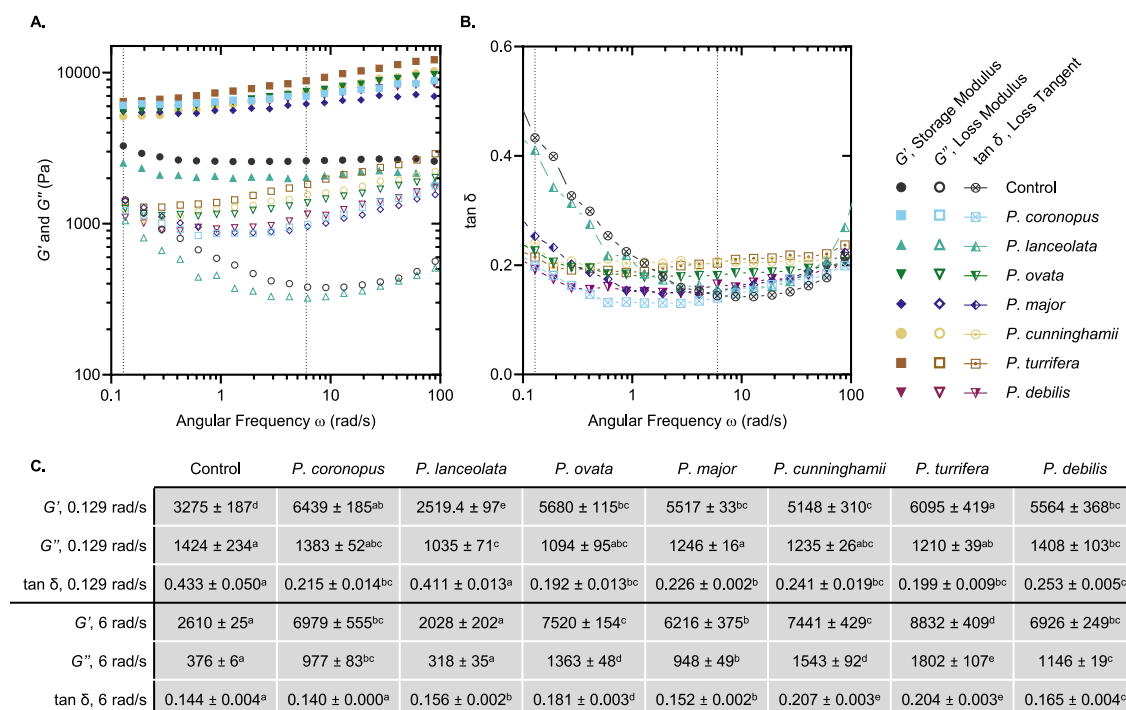


Fig. 3. Variation in A. storage modulus, (G'), loss modulus (G'') and B. loss tangent ($\tan \delta$) with frequency of rice flour-based gluten free doughs upon the 4% addition of *Plantago* WSF. C. Dynamic moduli were extracted at a low frequency (0.129 rad/s) and middle frequency (6 rad/s) and marked with dotted vertical lines in A and B. The control dough is rice flour without the addition of *Plantago* flour. Plots are averages of three replicates. Values are means $\pm$ SD. Samples sharing the same superscript letter in a row are not significantly different ($p > 0.05$).

with frequency in control and *P. lanceolata* doughs suggest that they behave more like a suspension of solid particles where viscous behaviour is more prominent at low frequencies due to particle-particle interactions (Genovese, 2012). The matrix filled with suspended solid flour particles in liquid would allow particles to slide past one another and allow energy dissipation as friction at low frequency before allowing more particle-matrix interactions at higher frequencies. A 70:30 wheat/resistant starch dough showed similar behaviour as the gluten network was disrupted (Arp et al., 2018). At low frequency, $\tan \delta$ of the 70:30 dough was greater than lower substitution levels before reducing as frequency increased until $\tan \delta$ was lower than the other formulations at middle and high frequencies (Arp et al., 2018). In doughs containing *Plantago* WSF, the released mucilage likely creates a viscosified continuous matrix (Ren et al., 2020a) and reinforces the dough where gel structure then dominates, both increasing the dynamic moduli but also significantly reducing the frequency dependence. Further supporting this is the similar rheological behaviour between control and *P. lanceolata* doughs: the *P. lanceolata* dough still contains additional quantities of inert filler WSF particles, but lower quantities of mucilage to reinforce the matrix.

The effect of differences in dough rheology were assessed in practice by assessing the kinetics of dough growth during fermentation (Fig. 4) and subsequently, the quality of gluten-free breads produced. When the mould was removed, doughs containing *Plantago* WSF changed in rest spread area and proofed area to different degrees (Fig. 4). It should be noted that rest spread occurred within the first 5 min but were first quantified at the 10 min time point. The visual appearance of the doughs and quantification of the dough area over time are displayed in Fig. 4A and B, respectively. For visualisation and comparison purposes, the area of the doughs after rest spread and proofing growth are presented graphically in Fig. 4C. Dough without WSF added spread at rest by c.

38% while doughs with WSF spread by <25.56% (Table 2). Of the WSF-containing formulations, *P. lanceolata* spread the most (25.56%) followed by *P. coronopus*, *P. ovata* and *P. major* doughs (17.65–16.80%). Doughs containing WSF from native species *P. cunninghamii*, *P. turritifera* and *P. debilis* spread at rest the least (13.82%–9.60%). Similar trends were seen in proofing growth where high rest spread correlated with high proofing growth, however *P. major* doughs had lower proofing growth, being more like doughs with Australian native species WSF. Dough rheology was strongly correlated with dough proofing behaviour. The amount of rest spread was negatively correlated with storage modulus (G') (Fig. 6; $r = -0.88$) showing that elasticity impacts mechanical robustness of the dough under internal expansion. Kinetic parameters of proofing were calculated by the change in dough area over time (Fig. 4B) and the growth modelled using the Gompertz model (Tjørve & Tjørve, 2017; Viduarre-Ruiz et al., 2019) (Table 2). The Gompertz model had good agreement with the experimental data ($R^2 = 0.983$ – 0.999) showing it to be suitable to estimate kinetic parameters. The velocity of proofing growth was estimated in the model parameter $\mu_{\max}$, which was greatest in *P. cunninghamii*, *P. turritifera* and *P. debilis* and lowest in control and *P. lanceolata* doughs. Interestingly this contradicts the findings of Viduarre-Ruiz et al. (2019) who found that their most fluid formulation (greatest rest spread) had the greatest $\mu_{\max}$. We suggest that in our doughs with high elasticity, the internal pressure from CO_2 produced during proofing is higher in doughs that are not spreading so when internal pressure becomes sufficiently high, volume change is rapid. This also corresponded with the time to the inflection point of the growth curve (parameter X; the time at which the sigmoidal growth curve inflects) where the inflection occurred sooner (Table 2) as the growth rate was not dampened by the spread of the dough caused by the lack of elasticity. Importantly, similar effects are seen in dough proofed vertically in a measuring cylinder (Supplementary Fig. 4) where the

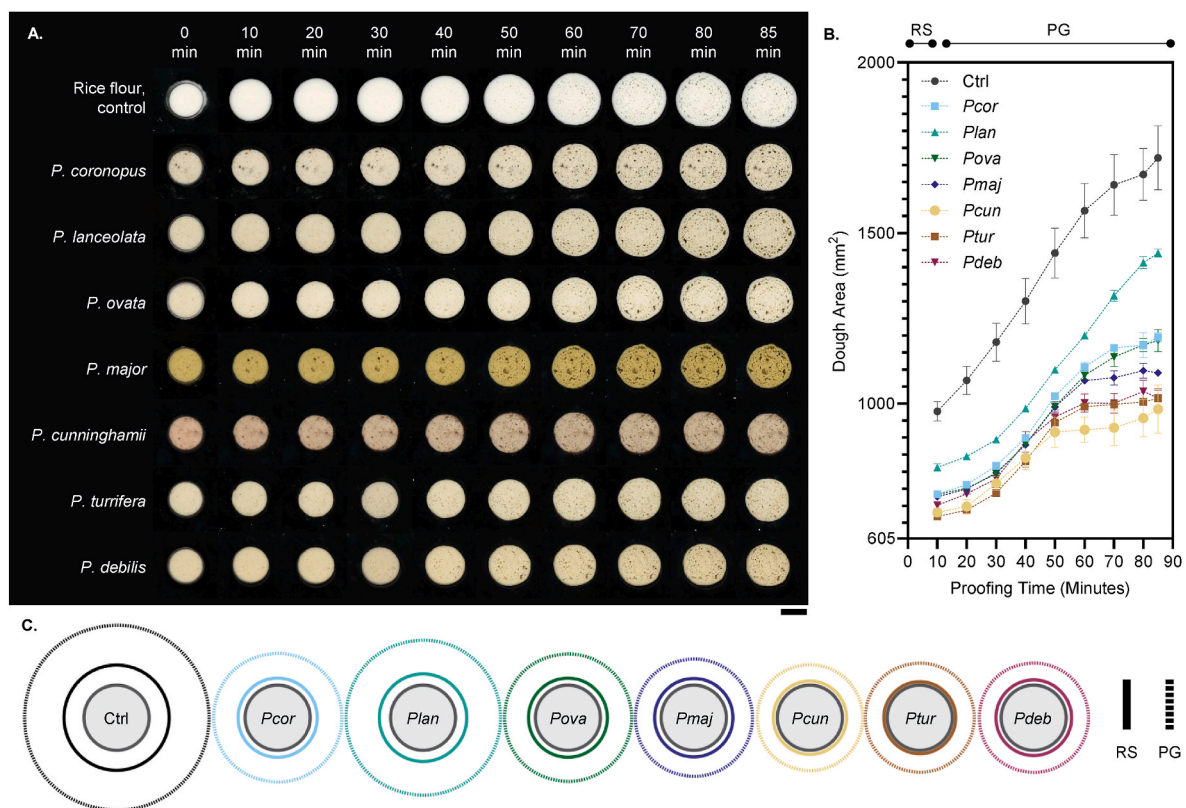


Fig. 4. Proofing dynamics of rice flour-based gluten free doughs upon the 4% addition of *Plantago* WSF. **A.** Representative images of changes in dough volume during proofing. Scale = 2 cm. **B.** Changes in dough area during proofing. Each dough began with an area of 605 mm² (size of mould) and change from 0 to 10 min represents rest spread (RS). Subsequent growth was considered proofing growth (PG). **C.** Schematic representation of changes in dough area after rest spread (RS) and proofing growth (PG). Grey circles indicate the mould size (Ø 22 mm) and area of dough at 0 min (605 mm²).

Table 2

Proofing kinetic parameters of rice flour-based gluten free doughs upon the 4% addition of *Plantago* WSF. Values are means $\pm$ standard deviation. Samples sharing the same superscript letter in a row are not significantly different ($p > 0.05$).

Kinetic Parameter	Control	<i>P. coronopus</i>	<i>P. lanceolata</i>	<i>P. ovata</i>	<i>P. major</i>	<i>P. cunninghamii</i>	<i>P. turrifera</i>	<i>P. debilis</i>
Rest Spread (%)	38.04 $\pm$ 1.82 ^f	17.65 $\pm$ 0.76 ^d	25.56 $\pm$ 1.00 ^e	17.54 $\pm$ 0.61 ^d	16.80 $\pm$ 1.12 ^{cd}	11.12 $\pm$ 1.68 ^{ab}	9.60 $\pm$ 0.26 ^a	13.82 $\pm$ 0.11 ^{bc}
Proofing Growth (%)	43.14 $\pm$ 1.52 ^d	38.50 $\pm$ 1.25 ^{cd}	43.61 $\pm$ 0.44 ^d	38.02 $\pm$ 2.14 ^{bc}	33.30 $\pm$ 1.05 ^{ab}	30.61 $\pm$ 3.91 ^a	33.99 $\pm$ 1.51 ^{ab}	30.89 $\pm$ 1.78 ^a
RS:PG	0.882	0.458	0.586	0.461	0.504	0.363	0.282	0.447
$\mu_{\max}$	0.046 $\pm$ 0.006 ^{de}	0.067 $\pm$ 0.007 ^{bcd}	0.041 $\pm$ 0.004 ^e	0.064 $\pm$ 0.002 ^{cde}	0.085 $\pm$ 0.001 ^{abc}	0.078 $\pm$ 0.011 ^{abc}	0.097 $\pm$ 0.017 ^a	0.091 $\pm$ 0.012 ^{ab}
<i>X</i> (min)	35.81 $\pm$ 2.44 ^{cde}	40.29 $\pm$ 0.63 ^{bc}	50.47 $\pm$ 3.19 ^a	42.44 $\pm$ 0.97 ^b	38.25 $\pm$ 0.39 ^{bed}	31.31 $\pm$ 2.47 ^e	36.58 $\pm$ 2.05 ^{cde}	34.00 $\pm$ 2.06 ^{cde}
Asymptote (mm ²)	1791.71 $\pm$ 97.08 ^a	1214.62 $\pm$ 30.36 ^c	1600.38 $\pm$ 61.54 ^b	1211.16 $\pm$ 34.18 ^c	1103.74 $\pm$ 4.34 ^{cd}	981.82 $\pm$ 64.63 ^d	1017.81 $\pm$ 18.24 ^d	1026.21 $\pm$ 31.89 ^d
Gompertz Model Fit (R ²)	0.994	0.991	0.997	0.999	0.983	0.991	0.991	0.990

volume of control and *P. lanceolata* doughs began to increase sooner and had a more gradual rate of increase compared to other formulations. The horizontal dough growth in the experiment in Fig. 4 allowed kinetics to be easily measured, but dough collapse was not possible despite being an important consideration. Importantly, the data obtained from this experiment agrees with data from vertically proofed doughs tested during breadmaking experiments (Fig. 6, $r = 0.78$) highlighting the suitability of this simple method for studying proofing kinetics. One advantage of also measuring vertically-proofed doughs at the time of baking is that a stability metric for collapse can be obtained. From this data we obtained a stability index (SI; maximum volume reached vs final volume; Supplementary Fig. 4) and found that fast proofing doughs like control and *P. lanceolata* doughs (Fig. 4, Supplementary Fig. 4) were more susceptible to collapse (SI = −110% and −123.8% respectively) than other formulations (SI = −18.6–0%). Many authors have concluded that if volume expansion in gluten-free doughs is not controlled, either by controlling fermentation time or altering the rheological properties, the doughs will proof too rapidly and succumb to collapse under gravity (Cappa et al., 2016; Kumar et al., 2018; Lazaridou et al., 2007; Mir et al., 2016). The development of *P. ovata*, *P. major*, *P. cunninghamii*, *P. turrifera* and *P. debilis* doughs were successful with no substantial collapse during proofing, while control and *P. lanceolata* doughs had significant rapid collapse and *P. coronopus* had slight collapse (negative stability indices) (Supplementary Fig. 4). In line with this, stability indices from vertically proofed doughs were strongly correlated with horizontally proofed parameters including rest spread, proofing growth (positive; less stable doughs = more rest spread and proofing growth), and $\mu_{\max}$ (negative; less stable doughs = faster growth), and parameters from rheology experiments including a very strong negative correlation with G' (less stable doughs = lower G') in the mid-frequency range where gel structuring dominates (Fig. 6).

As the doughs in rheology and proofing experiments are uncooked, effects observed in RVA experiments that we hypothesise to relate to co-polymer network formation with amylose are unlikely to contribute to the effects described before cooking. However, we believe that more than one mechanism can occur at different points in the breadmaking process, acting synergistically to improve final bread qualities. We hypothesise that the viscoelastic changes in doughs before cooking (when granules are still intact) are directly a result of particles of *Plantago* WSF becoming hydrated, with the mucilage becoming swollen and occupying intergranular spaces. The mucilage heteroxylan, remaining anchored to the WSF particles by the mucilage envelope, may begin to interact with heteroxylan from other WSF particles forming the mucin-like interactions described by Yu et al. (2017). These interactions, along with the occupation of intergranular space by the WSF particles, may act to reinforce the flour matrix leading to improved rheological properties, especially the frequency independence of $\tan \delta$. This is further exemplified by the control-like rheological properties of *P. lanceolata* doughs, as these still contains additional quantities of inert filler WSF particles, but minimal quantities of mucilage to reinforce the

matrix and buffer the intergranular spaces and reduce fluid-like behaviour from mobile granules slipping past each other at low frequency. The similar rheological behaviour between remaining formulations and slight but significant differences in G' and $\tan \delta$ particularly are then almost certainly a result of heteroxylan structure-function differences between the species. Importantly, co-polymer network formation may still occur during baking of breads when the granules exude their contents into the matrix where mucilage polysaccharides are already present.

3.4. Improved rheological and proofing behaviour of doughs containing *Plantago* WSF leads to improved gluten-free bread quality

The rheological behaviour influencing the proofing behaviour in turn influenced the final gluten-free bread quality. The structure of gluten-free loaves and their cellularity is shown in Fig. 5 and baking quality parameters, texture profile analysis and colour parameters are shown in Table 3.

The control and *P. coronopus* loaves have a low, flat-topped profile indicating collapse during proofing, while the *P. lanceolata* and *P. major* loaves have a 'horned' appearance more characteristic of collapse during baking (Fig. 5A). *P. ovata*, *P. cunninghamii*, *P. turrifera* and *P. debilis* loaves all maintained high profiles with domed tops, however *P. debilis* loaves did show slight evidence of collapse-related bowing not present in *P. ovata*, *P. cunninghamii* or *P. turrifera* loaves (Fig. 5A). The high-profile loaves also had few holes and small alveoli, while the low profile and 'horned' loaves had substantial holes and large alveoli, particularly in *P. major* (Fig. 5B). Collapsed loaves also show evidence of alveolar coalescence having extensive alveolar border irregularity, again, particularly noticeable in *P. major* loaves (Fig. 5B). Collapse resistance is critical to crumb formation as it influences many downstream qualities including volume. Specific volume is a key parameter to be improved in gluten-free formulations as it has a strong influence on consumer choice related to perception of freshness and texture (Monteiro et al., 2021). As such, rheological parameters, and subsequently proofing behaviour and control of collapse, were strongly correlated with bread quality characteristics (Fig. 6) including actual loaf volume and specific volume. Specific volume and actual volume were improved in formulations containing *Plantago* WSF (Table 3), which is generally at odds with previously published research. It was previously reported that a 5% replacement with *P. ovata* WSF did not significantly increase the specific volume of RF-based GF bread while *P. psyllium* WSF only slightly increased specific volume (Ziemichód et al., 2018). The differences reported here are attributed to formulation differences, with hydration level likely being a key factor. Baking loss describes the dough mass lost as CO₂ and water during proofing and baking and is a strong indicator of gas retention (Alvarez-Jubete et al., 2009; Gan et al., 1990). In doughs with significant collapse (Supplementary Fig. 4), baking loss is high due to mass ejection of gases during the collapse event. The greatest baking loss was therefore seen in control, *P. coronopus* and *P. lanceolata* loaves

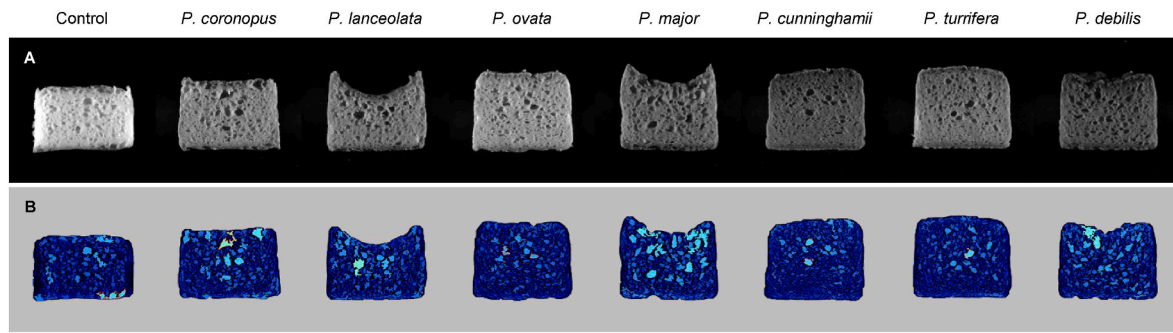


Fig. 5. Representative images of rice flour-based gluten free doughs upon the 4% addition of *Plantago* WSF flour. **A** Raw images of mid-loaf slices. **B** C-Cell cellularity of the same slice. Scale = 2 cm.

which have evidence of significant collapse and low specific volume (Table 3). The loaves that had no significant collapse, *P. ovata*, *P. cunninghamii* and *P. turrifera* (Fig. 5), correspondingly had the lowest bake loss (Table 3). As such, baking loss was strongly negatively correlated with both specific volume and actual volume (Fig. 6; $r = -0.74$ and $r = -0.86$, respectively). Adding to this, loaves containing *Plantago* WSF had significantly higher moisture content than the control (Table 3), but moisture content was similar between formulations indicating that most of the baking loss was through ejection of CO_2 . Alveolar formation and coalescence can be observed through the average alveolar diameter of the crumb and thickness of intraalveolar walls (Table 3). *P. cunninghamii* and *P. turrifera* loaves had alveoli 29% smaller than the control while loaves containing *P. major* WSF had alveoli that were 36% larger. Coalescence, particularly from rapid collapse, leads to thickened walls between the already enlarged alveoli. The thickness of the intraalveolar walls is therefore correlated with their average diameter (Fig. 6; $r = 0.77$). The loaf with largest alveoli, *P. major* had the thickest walls while *P. cunninghamii* and *P. turrifera* with the smallest alveoli had the thinnest walls.

Interestingly, differences in bread qualities like specific volume and texture were not significantly correlated with the crumb structure parameters assessed here (Table 3; Fig. 6). The link between crumb structure and volume is logical as the same mass of dough proofed into a bread of larger volume must have the crumb diluted across the same space. It is likely that *P. major* disrupts this correlation as despite its high specific volume, the loaves experienced significant collapse, and thus there is large spatial variation in porosity which may be too simply summarised in the average alveolar diameter. Indeed, the most successfully proofed loaves (not necessarily reflected in the specific volume), containing *P. ovata*, *P. cunninghamii* and *P. turrifera* WSF, had the smallest pores. At odds with published work (Ren et al., 2020c), top concavity does not describe collapse well as loaves with low concavity ($>1.17\%$) represented both completely collapsed crumb (control and *P. coronopus*) and resistance to collapse (e.g. *P. cunninghamii*, *P. ovata* and *P. turrifera*), with concavity only being significant in partially collapsed, ‘horned’ loaves (Table 3). Altogether, crumb structuring, especially in gluten-free bread, is complex and future work should consider the maximum proofed loaf height and subsequent stability indices along with porosity distribution to better study the relationship between crumb structure and bread quality.

Inconsistent or poor crumb structure leads to poor textural characteristics. The results of texture profile analysis (TPA) of the gluten-free bread crumb are presented in Table 3. Hard crumb is typical of poorly leavened GF breads and is perceived as staleness and thus unappealing, so low hardness is ideal. Crumb hardness varied from the control with some formulations being harder (e.g. *P. coronopus*) and some being softer than control (e.g. *P. turrifera*) (Table 3), though only one was significantly different. Crumb hardness was not significantly altered from the control in any formulation except for *P. turrifera* where crumb hardness was 18.2% lower. The moderate crumb hardness observed

even in leavened loaves is likely an effect of the highly-hydrophilic *Plantago* mucilage (Cowley et al., 2021) binding water and reducing the effective dough water content. It has often been reported that GF formulations containing the highly-hydrophilic psyllium mucilage are particularly susceptible to this (Cappa et al., 2013; Fratelli et al., 2018; Mancebo et al., 2015). A more effective bread rise leading to dilution of crumb within the loaf area, however, would ameliorate this effect creating a softer crumb even under insufficient hydration. This explains the lower crumb hardness in *P. ovata*, *P. cunninghamii* and *P. turrifera* breads, the loaf with the greatest volume (Table 3) and most effectively proofed structure (Fig. 5). Furthermore, *P. coronopus* and *P. major* loaves exhibited the greatest hardness resulting from the most extensive collapse. Control and *P. lanceolata* loaves also had significant collapse but *P. lanceolata* WSF contains less mucilaginous polysaccharides than *P. coronopus* and *P. major* (Cowley et al., 2021). *P. coronopus* and *P. major* loaves would therefore have a greater hydration deficit, in addition to extensive collapse, compounding the high hardness in comparison to *P. lanceolata* or control loaves. Evidence of water binding and rheological behaviour observed in this study point to the mucilage polysaccharides of *Plantago* WSF providing some of the internal cohesion properties that gluten would typically provide to the starchy matrix of breads. Indeed, the control bread had low cohesiveness (34.45%) compared with other formulations that were significantly more cohesive (Table 3). *P. major*, *P. cunninghamii* and *P. turrifera* loaves had the highest level of cohesion, up to 78% higher than control. Secondary TPA parameters like springiness and resilience, which represent a product’s ability to reform its original geometry after compression, were significantly improved from control in all formulations but *P. coronopus* for springiness. The control bread has a relatively low resilience (20.58%) compared with the WSF-containing formulations which were significantly higher (25.91–36.74%) indicating they resisted more permanent structural changes from deformation which is likely to be related to the improved internal cohesion (Table 3). Chewiness considers the combined effects of hardness, cohesiveness, and springiness to define the energy required to chew and break down a product for swallowing, which is typically lacking in GF products. Compared with control, chewiness was much higher in all WSF-containing formulations (46.7–96.8% higher) though there were few significant differences between these. Like with the other TPA parameters, chewiness was highest in *P. major* and *P. cunninghamii* loaves, though *P. turrifera* was lower in this parameter due to the reduced hardness.

Psyllium is particularly effective at imbuing products with improved internal cohesion and subsequent textural improvements (Franco & Manuel, 2022; Ren et al., 2020a). Ziemichód et al. (2018) and Pejcz and Burešová (2022) reported that *P. ovata* WSF- and *P. psyllium* WSF-augmented GF breads had high cohesiveness and chewiness compared to control without hydrocolloids added. This agrees with this study where cohesiveness, springiness, resilience and chewiness were generally significantly improved in all *Plantago*-containing formulations (Table 3). The high level of cohesiveness, chewiness, resilience and

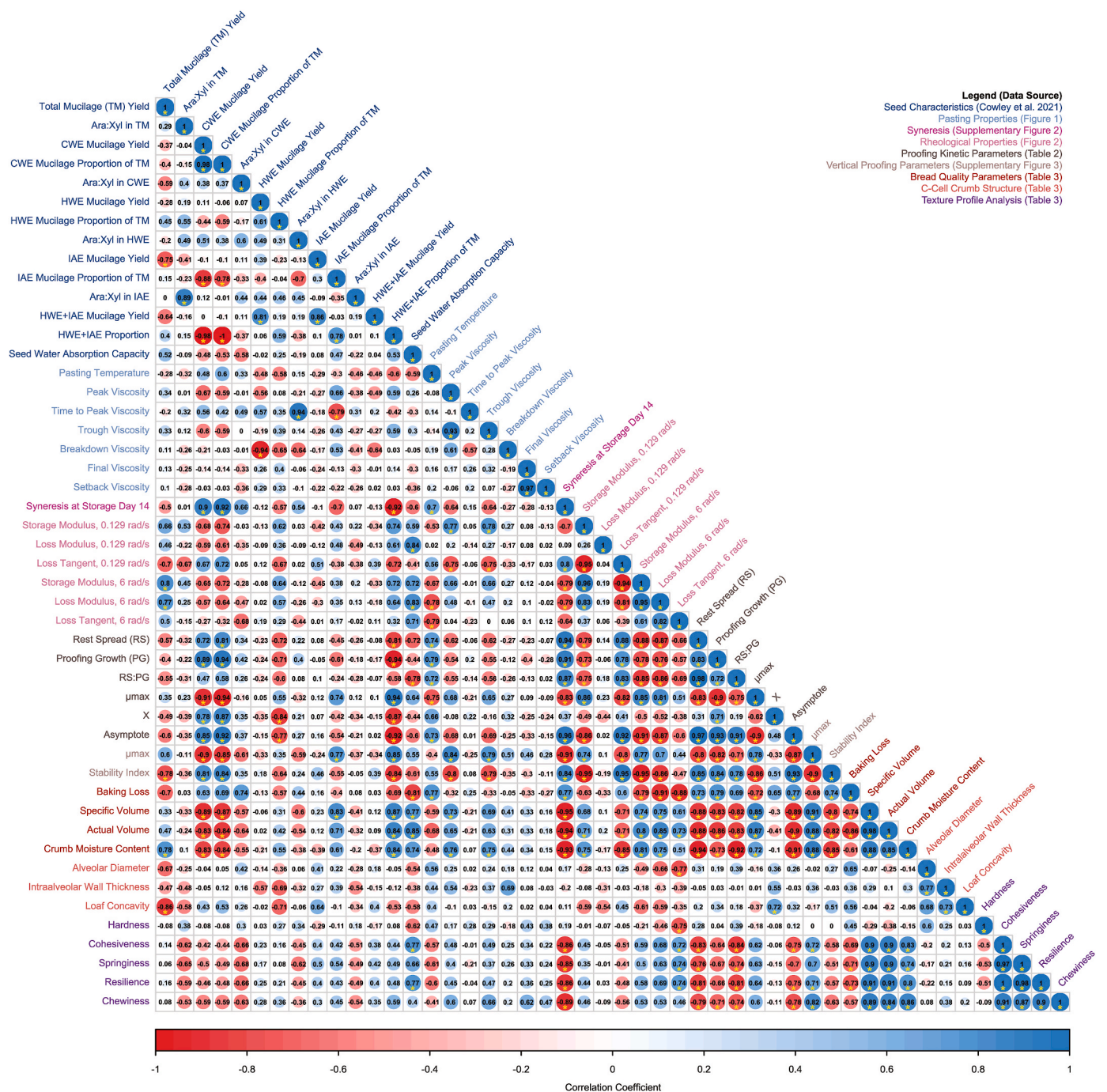


Fig. 6. Correlation matrix of key parameters tested here. Each cell shows the Pearson correlation coefficient and a circle graphically representing the strength (larger = stronger correlation) and direction (blue and red = positive and negative correlations, respectively) of the correlation. Statistically significant correlations ($p < 0.05$) are marked with a yellow asterisk below the correlation coefficient. Correlated parameter labels are colour-coded by analysis type and source (published literature Cowley et al., 2021 or figure/table in this study).

springiness in *Plantago* WSF-containing breads, particularly *P. cunninghamii* and *P. turrifera* breads, is attributed to system structuring (Haque & Morris, 1994). We suggest that network formation that is enhanced by Australian native *Plantago* species in RVA experiments (Fig. 2) may be specifically contributing to the high quality of GF breads made using Australian native *Plantago* species WSF, despite the reduced mucilage content compared to *P. ovata*. As mentioned in Section 3.1 and 3.2, it is possible that the co-polymer network formation may be contributing to system structuring that reduced syneresis after cooking (Supplementary Fig. 3). In Section 3.3, we suggested that rheological improvements in uncooked doughs are via a different mechanism that

would not preclude network formation when the doughs become cooked and the rice flour releases its amylose. Overall, *P. cunninghamii*, *P. debilis* and *P. turrifera* (and *P. bellidioides*) showed the strongest evidence of an interaction with cooking starch (reduced PT, enriched first-stage granule swelling, and gelation on cooling). These effects were not evident in other species tested and interestingly might be directly linked to the improved bread qualities we observed and why the effects seen must be at least partly independent of the WSF mucilage content (Fig. 6). Despite all having a lower mucilage content than *P. ovata* (Cowley et al., 2021), *P. cunninghamii*, *P. turrifera* and *P. debilis* all produced breads with comparable or superior qualities like specific and actual volume and

Table 3

Quality characteristics, texture profile analysis (TPA) and colour of rice flour-based gluten free doughs upon the 4% addition of *Plantago* WSF flour. Values are means $\pm$ standard deviation. Samples sharing the same superscript letter in a row are not significantly different ($p > 0.05$).

	Control	<i>P. coronopus</i>	<i>P. lanceolata</i>	<i>P. ovata</i>	<i>P. major</i>	<i>P. cunninghamii</i>	<i>P. turrifera</i>	<i>P. debilis</i>
Bread Quality Parameters								
Baking Loss (%)	21.58 $\pm$ 0.8 ^d	21.59 $\pm$ 0.06 ^d	22.88 $\pm$ 0.42 ^d	18.64 $\pm$ 0.58 ^{bc}	19.39 $\pm$ 0.04 ^c	16.35 $\pm$ 0.02 ^a	17.24 $\pm$ 0.12 ^{ab}	19.75 $\pm$ 0.05 ^c
Crumb Moisture Content (%)	53.77 $\pm$ 0.05 ^a	55.57 $\pm$ 0.01 ^c	55.05 $\pm$ 0.02 ^b	55.88 $\pm$ 0.07 ^c	55.92 $\pm$ 0.18 ^c	55.82 $\pm$ 0.04 ^c	55.86 $\pm$ 0.07 ^c	55.70 $\pm$ 0.11 ^c
Specific Volume (mL/g)	1.479 $\pm$ 0.023 ^a	1.666 $\pm$ 0.005 ^b	1.665 $\pm$ 0.001 ^b	1.805 $\pm$ 0.004 ^c	1.976 $\pm$ 0.006 ^d	1.877 $\pm$ 0.047 ^{cd}	1.946 $\pm$ 0.006 ^d	1.851 $\pm$ 0.037 ^{cd}
Actual Volume (mL)	236.03 $\pm$ 5.19 ^a	263.79 $\pm$ 0.56 ^b	257.41 $\pm$ 0.20 ^b	294.07 $\pm$ 0.83 ^c	317.16 $\pm$ 0.43 ^d	314.68 $\pm$ 7.11 ^d	324.68 $\pm$ 0.69 ^d	295.47 $\pm$ 7.04 ^c
Top Concavity (%)	0.70 $\pm$ 0.08 ^c	1.17 $\pm$ 0.62 ^c	15.65 $\pm$ 1.91 ^a	1.06 $\pm$ 0.08 ^c	7.67 $\pm$ 2.6 ^b	0.52 $\pm$ 0.02 ^c	0.45 $\pm$ 0.13 ^c	4.69 $\pm$ 0.44 ^{bc}
Average Alveoli Diameter (mm)	2.280 $\pm$ 0.006 ^{bc}	2.369 $\pm$ 0.108 ^{bc}	2.690 $\pm$ 0.145 ^{cd}	1.847 $\pm$ 0.029 ^{ab}	3.108 $\pm$ 0.238 ^d	1.623 $\pm$ 0.081 ^a	1.709 $\pm$ 0.019 ^a	2.552 $\pm$ 0.150 ^{cd}
Average Intraalveolar Wall Thickness (mm)	0.457 $\pm$ 0.003 ^{ab}	0.479 $\pm$ 0.003 ^c	0.524 $\pm$ 0.010 ^{de}	0.505 $\pm$ 0.005 ^{cd}	0.540 $\pm$ 0.014 ^e	0.448 $\pm$ 0.004 ^a	0.469 $\pm$ 0.001 ^{ab}	0.513 $\pm$ 0.000 ^{de}
TPA Parameters								
Hardness (g)	4370 $\pm$ 296 ^a	4672 $\pm$ 157 ^a	4062 $\pm$ 157 ^{ab}	4136 $\pm$ 68 ^{ab}	4540 $\pm$ 249 ^a	4159 $\pm$ 200 ^{ab}	3575 $\pm$ 182 ^b	4420 $\pm$ 118 ^a
Cohesiveness (%)	34.45 $\pm$ 1.36 ^d	45.35 $\pm$ 0.78 ^c	52.63 $\pm$ 1.03 ^b	52.50 $\pm$ 0.42 ^b	58.60 $\pm$ 0.92 ^a	60.18 $\pm$ 1.87 ^a	61.40 $\pm$ 0.14 ^a	49.85 $\pm$ 1.41 ^b
Springiness (%)	86.76 $\pm$ 0.39 ^d	88.05 $\pm$ 1.17 ^d	91.83 $\pm$ 0.68 ^{bc}	91.44 $\pm$ 0.50 ^c	93.91 $\pm$ 0.06 ^{ab}	94.37 $\pm$ 0.69 ^a	94.53 $\pm$ 0.54 ^a	91.65 $\pm$ 0.2 ^{bc}
Resilience (%)	20.58 $\pm$ 0.81 ^d	25.91 $\pm$ 0.07 ^c	30.46 $\pm$ 0.5 ^b	30.49 $\pm$ 0.17 ^b	35.02 $\pm$ 0.41 ^a	36.21 $\pm$ 0.73 ^a	36.74 $\pm$ 0.02 ^a	29.11 $\pm$ 0.23 ^b
Chewiness	1268 $\pm$ 165 ^c	1860 $\pm$ 74 ^b	1971 $\pm$ 4 ^b	1984 $\pm$ 35 ^{ab}	2495 $\pm$ 182 ^a	2365 $\pm$ 202 ^{ab}	2078 $\pm$ 97 ^{ab}	2010 $\pm$ 126 ^{ab}
Colour Parameters								
L*	51.79 $\pm$ 0.69 ^d	46.39 $\pm$ 1.94 ^c	49.88 $\pm$ 0.08 ^{cd}	49.98 $\pm$ 1.03 ^{cd}	40.13 $\pm$ 1.60 ^b	38.02 $\pm$ 0.29 ^b	47.32 $\pm$ 1.43 ^{cd}	32.34 $\pm$ 0.57 ^a
ΔL^*	–	–5.41	–1.91	–1.81	–11.66	–13.77	–4.48	–19.45
a*	–1.21 $\pm$ 0.10 ^a	1.43 $\pm$ 0.05 ^b	2.82 $\pm$ 0.07 ^d	6.66 $\pm$ 0.15 ^f	2.13 $\pm$ 0.04 ^c	5.85 $\pm$ 0.03 ^c	1.43 $\pm$ 0.05 ^b	6.88 $\pm$ 0.08 ^f
Δa^*	–	2.64	4.03	7.87	3.34	7.06	2.64	8.09
b*	4.13 $\pm$ 0.19 ^b	6.99 $\pm$ 0.29 ^d	5.56 $\pm$ 0.34 ^c	7.42 $\pm$ 0.08 ^d	11.01 $\pm$ 0.50 ^e	3.98 $\pm$ 0.04 ^b	5.02 $\pm$ 0.26 ^{bc}	0.93 $\pm$ 0.10 ^a
Δb^*	–	2.87	1.44	3.29	6.89	–0.15	0.89	–3.20
ΔE^*	–	9.67	6.57	10.55	13.12	16.14	5.20	20.92
Hue Angle (°)	73.67	78.44	63.11	48.09	79.05	34.23	74.10	7.70
Hue Family	Cool Yellow	Cool Yellow	Mid Yellow	Warm Yellow	Cool Yellow	Orange	Cool Yellow	Warm Red

texture (Table 3). For example, *P. turrifera* has 25% less mucilage than *P. ovata*, yet it produced more rheologically robust doughs and more voluminous breads with better texture. Furthermore, despite its evidence of collapse, *P. debilis* breads still retained positive attributes including a similar specific and actual volume and textural attributes to *P. ovata* bread. Therefore, it is possible that *P. debilis* mucilage content (lowest of all three Australian native species used in baking experiments) is slightly too low to meet a particular threshold that ensures subsequent quality like in *P. turrifera* or *P. cunninghamii*. It would be interesting to test this hypothesis by evaluating the performance of *P. bellidioides* WSF in GF dough and baking quality. We hypothesise that *P. bellidioides* may produce dough and breads with similar or better quality to even *P. cunninghamii* and *P. turrifera* considering (1) similar water absorption capacities (Cowley et al., 2021), (2) similar mucilage content, chemistry (i.e. A:X) and fractionation behaviour (i.e. larger share of gel-like mucilage fractions) (Cowley et al., 2021), and (3) even greater degrees of first-stage granule swelling and gelation in RVA experiments (Fig. 2). Additionally, future work should employ the use of farinograph (or similar) to account for differences in water absorption and how that subsequently affects final product quality (Arp et al., 2018; Pejcz & Burešová, 2022; Pejcz et al., 2018).

3.5. *Plantago* WSF's effect on product colour might influence commercial potential

While the addition of *Plantago* WSF lead to improvements in bread qualities and texture, the impacts on colour are also important as this can influence consumer acceptance. *Plantago* WSF generally darkened and reddened the bread crumb (Table 3) in agreeance with work by

others where phenolic compounds from the inner seed tissues were thought to undergo enzymatic browning during baking (Hadidy, 2020; Pejcz et al., 2018; Ziemichód et al., 2018). The polished white rice flour used is bright white in colour while the whole grain *Plantago* WSFs are various shades of brown (Supplementary Fig. 5A) and significantly darker (Supplementary Fig. 5B). Correspondingly, the addition of *Plantago* WSF darkened all formulations after baking (L^* , $\Delta L^* \neq 0$), though only significantly in *P. coronopus*, *P. major*, *P. cunninghamii* and *P. debilis* loaves. The addition of *Plantago* WSF significantly reddened the crumb colour ($\Delta a^* > 0$) with the greatest change being seen in *P. debilis*, *P. cunninghamii* and *P. ovata*. The effect of *Plantago* WSF addition on Δb^* had more range of effects than Δa^* with formulations showing both yellow and blue tones ($\Delta b^* > 0$, e.g. *P. major* and $\Delta b^* < 0$, e.g. *P. cunninghamii*). The value of ΔE^* displays the total perceptible colour difference between the samples and the control while the hue angle is used to interpret the hue family to broadly class the crumb colour (Table 3). The crumb colour of *P. turrifera* was most similar to the control, also a cool yellow hue, while *P. debilis* was starkly different, a purplish warm red. This is interesting given that raw *P. turrifera* and *P. debilis* WSFs are similarly light in colour (Supplementary Figs. 5A and 5B). Depending on the product being produced, the impact of colour change is not always negative. Gluten-free breads made from purified starches are lighter in colour than white wheat bread and thus natural 'earthy' colours from additives can be desirable (Gallagher et al., 2003). It is also known that colours, particularly those perceived as natural, influence expectation of sensory experience and perceived and actual nutritional value and this may influence consumer acceptability (Stintzing & Carle, 2004; Wei et al., 2012). Regardless, the colour changes might limit future applications so in pairs with similar impacts

on texture but different colour impacts (like *P. cunninghamii* vs *P. turrifera*), the species with lesser impact on colour might have more commercial potential. Sensory analysis will be an essential tool to ultimately determine consumer acceptability of the combined organoleptic properties introduced by *Plantago* WSF use including texture and colour, along with flavour and aroma that were not determined in this work. Comparisons to gluten- and psyllium husk-containing benchmarks may be particularly useful moving forward in finished product development.

4. Conclusions

In this study we show that natural variation in mucilage within whole seed flour of different *Plantago* species is a powerful tool to cause a variety of changes to the pasting, storage and gluten-free breadmaking of rice flour. We show the key role mucilage plays in the functionality of WSF but demonstrate that the effects are at least partly independent of mucilage content. This shows that natural variation in mucilage structure is also an important determinant of the functionality of mucilage-containing WSFs. We hypothesise that the share of more gel-like fractions of mucilage with higher A:X in some WSFs (e.g. Australian native species like *P. turrifera*) might promote network formation and system structuring in starch-based systems that synergistically improves product quality despite lower mucilage contents. Indeed, the proportion, but interestingly *not* the content, of more gel-like fractions in the mucilage (hot water and intense agitation extractable fractions) were strongly correlated with many parameters explored here including dough proofing behaviour and bread qualities like specific volume. More work is needed to confirm the mechanisms by which *Plantago* mucilage polysaccharides differentially alter the properties of starch pastes and gluten-free breads, particularly to scrutinise the 'blockiness' of *Plantago* heteroxylan, subsequent helix formation, and the possible heterotypic interactions occurring. We suggest that discrete fractionation techniques and rheological analyses would be particularly informative as they have yielded great insight for *P. ovata* (Ren et al., 2020d; Yu et al., 2017). As there are over 200 *Plantago* species, the methods outlined here might be useful to screen the functionality of *Plantago* natural variants in the (Cowley et al., 2021; Herliana et al., 2023) to probe the structure-function relationship of their complex heteroxylans. Furthermore, this approach has highlighted that by scrutinising relatives of a commercially relevant species like *P. ovata*, an orphan crop plagued by agronomic constraints, we can identify species, like *P. turrifera*, that have potential as nutritious and sustainable hydrocolloid replacements and are adapted to grow in harsh Australian conditions.

CRediT authorship contribution statement

James M. Cowley: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Yi Ren:** Writing – review & editing, Methodology, Conceptualization. **Lucija Štrkalj:** Writing – review & editing, Conceptualization. **Tim J. Foster:** Writing – review & editing, Supervision, Methodology. **Rachel A. Burton:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

James Cowley and Rachel Burton have patent #WO/2022/256862 issued to The University of Adelaide. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2024.110788>.

Data availability

Data will be made available on request.

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- Ziemichód, A., Wójcik, M., & Różyło, R. (2018). Seeds of Plantago psyllium and Plantago ovata: Mineral composition, grinding, and use for gluten-free bread as substitutes for hydrocolloids. *Journal of Food Process Engineering*, Article e12931. <https://doi.org/10.1111/jfpe.12931>

Appendix II - Career and Research Skills Training (CaRST)

What is Career and Research Skills Training (CaRST)?

Career and Research Skills Training (CaRST) is a specialised training and development program for Higher Degree by Research (HDR) students at the University of Adelaide.

CaRST comprises the 'Development Component of the Structured Program' and student participation extends from enrolment to thesis submission. HDRs are required to complete a minimum number of CaRST hours by thesis submission: 120 hours for PhDs and 60 hours for MPhils. Activities must also be distributed across all four domains identified in the Researcher Development Framework from Vitae.

CaRST Record

Below is a record of the activities that I completed during my PhD Candidature as per the requirements of the Career and Research Skills Training (CaRST) program



THE UNIVERSITY
of ADELAIDE

This is to certify that

Ms Lucija Strkalj

has completed

120 hours

of Career and Research Skills Training

*Dr Monica Kerr,
Director, Career and Research Skills Training*

30 Sep 2024

Date

adelaide.edu.au

CRICOS Provider Number 00123M

seek LIGHT

Authorship statements

Following authorship statements are included as a requirement for PhD of University of Adelaide.

Statement of Authorship

Title of Paper	Structural and functional properties of fibre from psyllium (<i>P. ovata</i>) husk: current knowledge and strategies to expand its application in food and beyond
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

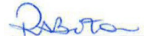
Principal Author

Name of Principal Author (Candidate)	Lucija Štrkalj		
Contribution to the Paper	Original draft, experimental work, conceptualization, writing – editing		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27/09/2024

Co-Author Contributions

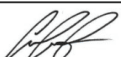
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- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Rachel A. Burton		
Contribution to the Paper	Conceptualization, writing – editing, supervision, funding acquisition		
Signature		Date	27/09/2024

Name of Co-Author	Gleb E. Yakubov		
Contribution to the Paper	Conceptualization, writing – editing, supervision, funding acquisition		
Signature		Date	27/09/2024

Please cut and paste additional co-author panels here as required.

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Statement of Authorship

Title of Paper	Fibre fractions from <i>Plantago ovata</i> mucilage show contrasting effects on structure and digestibility of corn starch with varying amylose content at different temperature treatments
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
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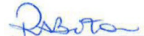
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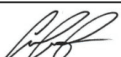
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Statement of Authorship

Title of Paper	Competitive gelation between starches and psyllium fibre (<i>Plantago ovata</i> mucilage): the effect of composite structure on rheology and freeze-thaw stability
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Publication Details	

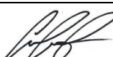
Principal Author

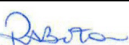
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Title of Paper	Rheological characterisation and enzymatic hydrolysis of composite rice starch- <i>Plantago</i> fibre gels
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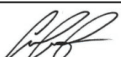
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Statement of Authorship

Title of Paper	Extraction, composition, and rheology of fibre fractions from four <i>Plantago</i> species with varying hydration and gelling profiles
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
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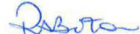
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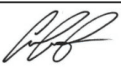
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Statement of Authorship

Title of Paper	Application of <i>P. ovata</i> and <i>P. turrifera</i> fibre in gluten-free bread reveals protective effects on storage stability but differences in bread quality
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
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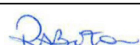
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Name of Co-Author	William J. Hirt		
Contribution to the Paper	Conceptualization, experimental work, writing – editing, supervision		
Signature		Date	27/09/2024

Name of Co-Author	Rachel A. Burton		
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