Analysis of *Plantago* species variants for novel functional and *in-vitro* fermentation properties

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Migelhewage Dinithi Tharuka Jayampathi Silva

09.09.2022

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This thesis is dedicated to my Amma (mum) and Thaththa (dad).

You are the reason for who I am today and for everything I'll ever be.

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CHAPTER 1

Thesis Introduction

Thesis introduction

Food manufacturing and nutraceuticals such as fibre supplements are extensive and ever-growing global industries where research and development are being constantly carried out to manufacture products with better quality, better sensory perception, and improved nutritional and health benefits. Hydrocolloids, which are hydrophilic long-chain polymers (polysaccharides and/or proteins) of various origins, both natural and artificial, are used in the food and fibre industry. Psyllium is one such hydrocolloid derived from the mucilaginous polysaccharides of *Plantago ovata* seeds and is used widely in the food industry, including as a fibre supplement due to its strong water-holding ability. Although there are many mucilage-producing members in the *Plantago* genus, *P. ovata* is the only species currently used commercially in the food and fibre industry as a source of mucilage. Despite the high demand, there are many constraints to obtaining a consistent supply of psyllium with good quality as the species is difficult to breed commercially and there is a little genetic variation to improve production for food functionality purposes. Furthermore, although *P. ovata* mucilage provides good faecal bulking and laxative properties, it is poorly fermentable thereby providing lesser fermentation-related health benefits and potentially making it a less-than-ideal fibre source. However, there may be novel sources of *Plantago* mucilage that can improve these constraints, such as variation generated through induced mutation, through processes such as fractionation, or found naturally, such as in other members of the *Plantago* genus that may be used as alternatives to *P. ovata* with similar or improved uses in food and fibre.

These alternative options raise questions about the possibility of novel or improved sources of mucilage from different species. Do the differences in mutant mucilage polysaccharide structure and composition caused by gamma irradiation *P. ovata* seeds provide better forms of psyllium for use as food hydrocolloids? Does whole mucilage of *P. ovata* provide the best functionality, or could fractionation of mucilage be used to tailor the properties of food? Could alterations to polysaccharide structure by mutation give rise to more fermentable mucilage with superior performance in faecal bulking as well as providing good amounts of beneficial compounds produced by fermentation in the gut? Are there better

fermentable species within Australian native *Plantago* that can be exploited for use in food and fibre applications? These research questions, that have shaped this thesis, are summarized in Figure 1-1.

Thesis structure/abstract

This thesis comprises six chapters: a general thesis introduction (Chapter 1), a literature review (Chapter 2), a conventional thesis chapter (Chapter 3), an unpublished research manuscript (Chapter 4) and a conventional thesis chapter (Chapter 5) with a final general discussion, including ideas for further research (Chapter 6). A schematic overview of the thesis structure is presented in Figure 1-2.

Chapter 3 is a conventional research chapter describing the screening process to identify potentially useful *P. ovata* mutants, with subsequent investigations into the differences in mucilage structure, composition, and food-related functionality. Dr Jana Phan (PhD submitted 2018) observed differences in the structure and composition of *P. ovata* mutants and studied mucilage development using a gamma-irradiated *P. ovata* mutant population developed in a previous project aiming to identify xylan synthase genes. Chao Ma and Dr James Cowley in their honours research work studied compositional and structural differences in mucilage polysaccharides of a selection of *P. ovata* mutants and their underlying gene mutations. Lina Herliana (PhD thesis submitted 2022) used *P. ovata* mutants to study seed and capsule development to identify improved forms of psyllium and identified a candidate mutant with increased mucilage production. Here in Chapter 3 of this thesis *P. ovata* mutants were studied to find out if differences in their mucilage properties caused by mutation can be exploited to identify novel forms of psyllium for use in food and fermentation applications. Mutants were screened to identify those with differences in mucilage composition and arrangement using monosaccharide analysis and microscopy followed by a study of functional properties including water absorption, influence on starch swelling, emulsification properties, foaming properties and alteration of the pasting properties of rice starch and rice flour when combined with a mucilage fraction. Although mutants displayed significant differences in mucilage composition, their functionality in altering pasting properties was only subtle when compared to the wild type. There is some literature available on the fermentation of *P. ovata* which shows that it is a

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poorly fermentable substrate, although it serves as a good stool-bulking agent in human fibre applications. In this study, the husk of four selected mutants and wild-type *P. ovata* and husk fraction material produced by the removal of the cold-water extractable fraction of the husk was tested *in vitro* to investigate if they can provide better fermentation and related benefits by increased production of short-chain fatty acids (SCFA) using mixed human faecal inoculum. Static *in vitro* fermentation with timed removals was carried out. An automatic gas reading system was used to monitor gas production and the changes in SCFA and ammonia and polysaccharides were studied throughout the course of fermentation using spectrophotometry and liquid-state nuclear magnetic resonance (NMR). It was found that there was a higher production of SCFA by the whole husk, in comparison to the cold-water fraction removed husk, however, mutant *P. ovata* only displayed subtle differences in fermentability when compared to the wild type.

Chapter 4 is an unpublished research manuscript where the influence of *P. ovata* seed fractions on starch pasting was investigated. Although there is published research available profiling fractions of *P. ovata* mucilage and its rheological properties, no work has been done to determine the functionality of such mucilage fractions in starch-based foods. In this study, changes to the pasting properties and preliminary baking performance of gluten-free rice flour formulations were studied after the addition of aqueous fractions of *P. ovata* whole-seed flour and husk. Different fractions led to distinct changes in the pasting profiles of rice flour and rice starch and altered the baking performance of model gluten-free bread. It was found that native forms of *P. ovata* seed (whole seed flour and husk) had better functionality than fractionated mucilage.

There is very limited research available on the composition and fermentation of native *Plantago* species. In his PhD, Dr James Cowley (submitted 2020) studied the seed composition and food-related functionality of diverse Australian native *Plantago* species. The work described in Chapter 5 complements this work by investigating differences in the fermentability of whole seed flour of nine naturalised and Australian native *Plantago* species, as well as the differences in the fermentability of the husk, de-husked seed and whole seed flour of the commercially used species *P. ovata,* using the same experimental

methods for *in vitro* fermentation as described in Chapter 3. Significant differences were found in the fermentability among the species, and the factions of *P. ovata* although all of them were poorly fermented overall.

The final chapter, Chapter 6, is a summary and general discussion of the findings of this research work and suggests future research directions.

Covid impact

The research work in this thesis was significantly affected by the Covid-19 pandemic and the impact of this is described here.

The PhD project started under the joint agreement PhD programme between the University of Adelaide, Australia (home university) and the University of Nottingham, UK (host university), where the candidate is expected to spend a minimum of 18-month study period at each institution for the joint award of the degree. The project was planned in a manner so that the candidate would spend the first 18 months at the University of Adelaide, the next 18 months in Nottingham and the final 12 months in Adelaide. The candidate's relocation to Nottingham was continuously delayed during the period of December 2019-December 2020 due to the high number of Covid-19 in the UK, and travel restrictions. Also, the candidate holds a foreign passport which did not allow the candidate to return to Australia once departed to the UK until travel restrictions were lifted in March 2022. The candidate had to withdraw from the joint PhD programme and the University of Nottingham due to the inability of meeting the award conditions and travel restrictions and became a candidate of the University of Adelaide only.

The PhD project was focused on studying the structure-functionality traits of *Plantago ovata* mucilage in food systems, including high-temperature rapid visco analysis, using the equipment and resources available at the University of Nottingham. However, the inability to relocate necessitated changing the focus of the project. This led to having to seek alternative paths to complete the project within Australia itself. Therefore, in February 2020 the candidate relocated to Queensland to collaborate with a group at

the University of Queensland where the research was focussed on *in-vitro* fermentation. However, during this period access to equipment like NMR and consumable deliveries were affected by Covid as well as the floods in Queensland. The scheduling of the *in-vitro* fermentation was affected by the Covid social distancing requirements and lockdowns as experiments require a team of 8-10 personnel to be working in close quarters.

Apart from the impact on the research project and candidature, the candidate's health was impacted by Covid-19. In September 2021, the candidate was hospitalized after displaying severe chest pain due to side effects of the second dose of the Pfizer Comirnaty vaccine and had to refrain from obtaining the booster vaccination. In January 2022, the candidate contracted Covid for the first time. In late May 2022, the candidate contracted Covid for a second time, followed by long Covid affecting the health and well-being of the candidate in the latter part of the PhD project.

Other unavoidable circumstances

The *Plantago ovata* mutant fermentation experiment described in Chapter 3 of this thesis was scheduled to run for 72 hours over 25th-28th May 2021, after much training, and preparation of reagents and substrates over a long period of time. A few hours after the inoculation of all samples and starting of the fermentation experiment Queensland was affected by a widespread power outage caused by a fire and explosion at Callide power station at Biloela, Queensland. The automatic gas reading systems and the incubators did not have supplementary power throughout this period. The automatic gas reading system had to be reset leading to a loss of all the gas data from the point of inoculation to the point when the equipment resetting occurred when the power was restored. The build-up of gas within the fermentation bottles affected the fermentation activity, retarding the growth of the bacteria during this period, and the recovered gas data was also affected rendering them unusable. Usable data is included in Chapter 3 but the power cut severely affected data quality and subsequent usefulness for supporting research outcomes.
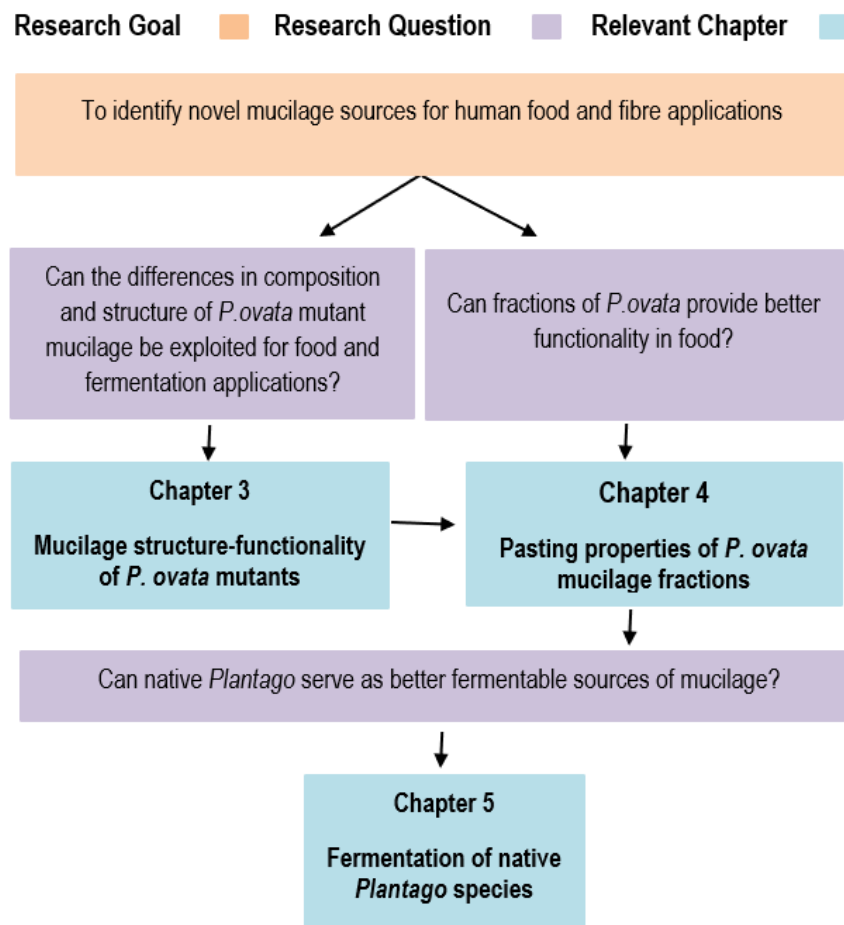
Figure 1-1. Schematic overview of the research questions that structured the thesis and their relevant research chapters

Chapter 1	**Thesis introduction** • Context of the study • Structure of the thesis	
Chapter 2	**Literature review** • Hydrocolloids in the food industry • Gastrointestinal fermentation • Psyllium – uses, need for improvement, novel forms	
Chapter 3	**Mucilage structure-functionality of *P. ovata* mutants** • Identifying *P. ovata* with different mucilage characteristics through screening • Investigating the structure and composition of mutants • Studying the food-related functional properties of mutants • Studying the fermentability of mutant mucilage over a 72h period using in vitro fermentation with human faecal inoculum • Investigation of the production of SCFA, NH3, and gas at timed intervals	Conventional research chapter
Chapter 4	**Pasting properties of *P. ovata* mucilage fractions** • Investigating the composition of *P. ovata* husk and whole seed flour mucilage fractions • Influence of fractions to altering the pasting properties of rice flour/starch • Preliminary investigation of the influence of fractions on gluten-free doughs	Publication format Target journal: *Food Hydrocolloids*
Chapter 5	***In vitro* fermentation of diverse *Plantago* seed-derived substrates** • Studying the fermentability of native *Plantago* over a 48h period using *in vitro* fermentation with human faecal inoculum • Investigation of the production of SCFA, NH_3, and gas at timed intervals	Conventional research chapter
Chapter 6	**General Discussion** • Summary • Future directions	
Appendix	• Sample details of mutant *P. ovata* fermentation • Sample details of diverse *Plantago* material fermentation • Career and Research Skills Training • Candidature milestones	

Figure 1-2. Schematic overview of the thesis structure.

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CHAPTER 2

Literature review

Hydrocolloids in the food industry

Hydrocolloids, comprised of hydrophilic proteins and polysaccharides, either in pure or mixed form, are widely used in the food industry to develop stability, texture and beneficial appearance in processed foods. They are long chain polymers that form gels/viscous dispersions when mixed with water. The presence of many hydroxyl groups makes them hydrophilic but their properties are intermediate between a suspension and a true solution, thus exhibiting properties of colloids, so they are called "hydrocolloids" (Saha and Bhattacharya, 2010). Hydrocolloids modify the rheology of foods by changing the mechanical solid properties and flow behaviour, thus affecting viscosity, texture and sensory properties and promoting properties such as thickness, sliminess, creaminess and smoothness of food (Malone *et al.*, 2003).

Hydrocolloids can be considered some of the most widely used ingredients in the food industry which are used in numerous food products such as jams, jellies, ice cream, candies, cakes, gelled desserts, salad dressing, sauces, toppings and soups (Milani and Maleki , 2012)*.* They are used for many purposes including as stabilizers, fat replacers, gelling agents, whipping agents, clouding agents, flocculating agents and emulsifiers. They are also applied as agents for inhibition of crystallization, to form edible films and as flavour encapsulates (Li and Nie, 2016). In the food industry, biopolymers are widely used as food additives to improve stability, texture and appearance and to change the rheological properties of food (Malone *et al.*, 2003). Table 2-1 summarizes some applications of hydrocolloids in foods.

When hydrocolloids are found in higher concentrations in solutions they interact with each other more often leading to an increase in the viscosity (Milani and Maleki, 2012) by forming an interactive entangled network (Graessley, 1973). The degree of viscosification depends on various factors such as temperature, pH, type of polymer and its concentration (Burey *et al.*, 2008). They are used to give thickness and texture to products such as sauces, gravies and desserts (Saha and Bhattacharya, 2010).

At sufficient concentration and conditions, long chain polymers form viscous gels in water and modify the solid properties and flow behaviour of foods (Milani and Maleki , 2012). Gel formation in most polymers occurs through cross linking forming a 3D network where junction zones are formed by aggregating of

interchain linkages. There are affected by various factors like the presence of ions in the medium, temperature and the hydrocolloid structure (Burey *et al.*, 2008) (Figure 2-1). Gelling is an attractive quality, as it can impart a soft but solid texture to foods which can be exploited for various applications in the food industry such as structuring and strengthening of food, texturizing, and as controlled release agents (Burey *et al.*, 2008). Food items such as jams and jellies get their desirable texture from the use of gelling agents such as pectin and gelatin (Saha and Bhattacharya, 2010).

Hydrocolloids are able to provide emulsification properties in mixtures due to their viscosifying nature by reducing the coalescence of dispersed droplets and slowing down phase separation (Wang *et al.*, 2011). Solid hydrocolloid particles can also stabilize mixtures by the formation of pickering emulsions (Murray, 2019). Cui *et al.*, (2021) defines a pickering emulsion as, "emulsions that are stabilized by an adsorbed layer of solid particles at the emulsion drop surface."

Stabilization of food items like ice cream is another application of hydrocolloids. The formation of ice crystals due to separation of water can cause a negative impact on the organoleptic properties of frozen food including their flavour and texture (Ghosh and Coupland, 2008). Hydrocolloids are used to immobilize the water which prevents them from separating and forming ice crystals thereby maintaining the creamy smooth texture which is desirable in frozen dairy desserts (Regand and Goff, 2003).

Emulsion food systems such as salad dressings, ice cream and mayonnaise acquire desirable rheological and organoleptic properties from the presence of integral fat (Chung *et al.*, 2013). However, concerns about the negative health effects of fats have increased the demand for reduced fat products (Hoefkens *et al.*, 2011, Weiss *et al.*, 2010). Thus, there is an increased demand for fat replacers and fat substitutes in the food industry, to impart similar properties to fat reduced products as found in full fat products (Chung *et al.*, 2013). "A fat replacer is an ingredient which may or may not provide nutritional value and replaces some or all functions of fat in a particular food system, while fat substitutes provide no energy and will be a replacement for all the functions of fat in a food" (Li and Nie, 2016). Fat replacement is an important application of food hydrocolloids, as reduction of dietary fat has become a driving dietary goal of many

consumers. Although consumers prefer food with minimal fat and calories, they also want the food to taste good, which has created a significant challenge to the food manufacturers (Yang *et al.*, 2006). In lower fat products, the oils and fat may be replaced by "structured water" which provides better eating qualities for reduced fat products (Milani and Maleki , 2012). Carbohydrate based fat replacers have displayed greater potential due to their multi-functional properties and they significantly improve the qualities of reduced fat products (Lim *et al.*, 2010). This is important because fat provides many functional properties to food, which affect the processing of products and their sensory acceptability and these functions have to be accounted for when reducing the fat content of products (Yang *et al.*, 2006). Hydrocolloids are used widely as fat replacers; for example xanthan gum as a thickener in Italian salad dressings; xanthan and guar gum as fat replacers in reduced fat mayonnaise; inulin in frozen desserts as a fat replacer with a fatty mouth feel and freeze thaw-stability (Milani and Maleki , 2012). Examples of hydrocolloid fat replacers include pectin, okra gum, guar gum, carrageenan, xanthan gum, sodium alginate, inulin, locust bean gum and gum tragacanth (Aziznia *et al.*, 2008). According to Chung *et al.*, (2013), some hydrocolloids, apart from being fat replacers, also increase the flavour intensity of fat-reduced emulsions by increasing the time that non-polar flavour molecules are retained in the food system. The fat content, and type of fat replacer used, significantly affects the functional properties of ice cream such as melting rates, viscosity, hardness and also the sensory properties (Karaca *et al.*, 2009). The largest number of fat replacers used in ice cream are carbohydrate based. These hydrocolloids possess functional properties such as gelling, thickening and emulsifying which provides flow properties and mouth feel similar to that of fat globules in an aqueous system. Use of natural gums as fat replacers in ice cream provides many benefits due to their low cost, availability and lack of toxicity (Javidi *et al.*, 2016).

Use in gluten-free products is another application of hydrocolloids in food (Anton and Artfield, 2008), which is gaining much interest due to the negative health effects on coeliac disease patients, by gluten, the structure-forming protein found in wheat. However, production of high quality starch-based baked products is quite challenging using gluten-free flour and there are growing applications of the inclusion of

hydrocolloids for improvement of structure, reduction of dehydration and crumb hardening in gluten-free foods (Gallagher *et al.*, 2004; Gómez *et al.*, 2007; Guarda *et al.*, 2004).

Hydrocolloids as gluten replacers

Importance of gluten in the food industry

Globally, bread is one of the most widely eaten food products, traditionally prepared using wheat flour. Wheat grains contain gluten, a structure-forming protein which when hydrated forms an extensible dough that has good gas holding capacity during the proofing process (Gallagher *et al.*, 2004) (Figure 2-2). This viscoelastic 3-D structure consists of a network of gluten which entraps granules of starch and traps gas bubbles, which then rise in the oven and form an solid structure which is porous (Hu *et al.*, 2021). Successfully-proofed breads have desirable volume, crumb structure, elastic properties, texture and appearance (Gallagher *et al.*, 2003). However, gluten causes health problems for celiac disease patients and an increasing number of non-celiac people with varying levels of gluten sensitivity are now choosing to actively avoid gluten. As the incidence of gluten intolerance and avoidance is increasing there is a rising demand for gluten-free foods. However the major challenge associated with these products is reproducing the structure of bread provided by gluten (Anton and Artfield, 2008).

Current methods of gluten replacement

Hydrocolloids, which include a large number of polysaccharides, have been tested for mimicking the structure of gluten (Anton and Artfield, 2008). Replacing gluten in bread is a significant technological challenge for the production of many high quality starch-based foods (Gallagher *et al.*, 2004). Researchers have used various gums, dietary fibres and enzymes to develop gluten-free formulations, including hydroxypropylmethylcellulose (HPMC), guar gum and carboxymethylcellulose (CMC) (Demirkesen *et al.*, 2010). Hydrocolloid addition has been used to give structure, prevent staling and extend the shelf life of gluten-free products by entrapping moisture within the crumb due to the high water holding capacity of the hydrocolloids, thereby preventing the drying out of crumb which causes a hard texture (Gómez *et al.*, 2007). HPMC, guar gum, locust bean gum and carrageenan are some of the most effective gum and

hydrocolloid agents for gluten replacement which are quite costly ingredients. Gums and hydrocolloids improve the appearance and texture of gluten-free products by improving dough consistency, gas retention and extension of the shelf life (Mariotti *et al*., 2009).

Problems associated with gluten-free products

The removal of gluten creates many technical problems for a baker, and as a result most gluten-free products on the market tend to have poor mouthfeel, flavour, and overall quality. Thus food scientists are interested in looking for alternatives to gluten that mimic some or all of its technological functions in gluten-free products (Gallagher *et al*., 2003). As suggested by Houben *et al.*, (2012) more investigation is necessary to provide celiac patients with high-quality gluten-free products. Also, there is an increasing demand for gluten-free products by non-celiac patients, which may hasten the research and development in this area, which in turn may lead to the reduction of market prices of gluten-free products. The global gluten free food market has been reported to be $5.72 billion in 2021 and is predicated to grow at a cumulative annual growth rate of 8.29% to reach a market size of $9.99 billion in 2028 (Fortune business insights, 2022).

 A study by Lee *et al.* (2007) identified that gluten-free food in the USA was not readily available and is more expensive than its gluten containing counterparts. With the increasing rates of celiac diseases, it is important to consider the economic burden that a gluten-free diet might create for gluten-sensitive persons. It is suggested that the higher cost, reduced nutrition, and lower availability may impose financial barriers that could have a strong negative effect on the quality of life of celiac patients in the USA. A study in the UK by Singh and Whelan, (2011) have also described similar findings, where a limited availability of gluten-free food, especially in smaller food businesses such as budget supermarkets and corner shops compared to larger super markets, was identified. Thus, research making gluten-free foods more accessible and affordable would support increased diet compliance and decrease the risk of negative effects of celiac disease.

Fermentation

Dietary fibre and gastrointestinal fermentation

Codex Alimentarius (2009) defines dietary fibre as "carbohydrate (CHO) polymers with ten or more monomeric units , which are not hydrolysed by the endogenous enzymes in the small intestine (SI) of humans and belong to the following categories: Edible CHO polymers naturally occurring in the food as consumed, CHO polymers, obtained from food raw material by physical, enzymatic, or chemical means or synthetic CHO polymers." European Food Safety Authority (EFSA) (2009) defines dietary fibre as "Non-digestible carbohydrates plus lignin, including all carbohydrate components occurring in foods that are non-digestible in the human small intestine and pass into the large intestine."

Ha, Jarvis and Mann, (2000) defines dietary fibre as ''any dietary component that reaches the colon without being absorbed in a healthy human gut." And classified them based on site of action; upper or lower gut and then their chemical nature, origin and the possibility of being utilized by microorganisms (Figure 2-3). Jiménez-Escrig & Sánchez-Muniz, (2000) state that "Dietary fibre is an overall description of mainly carbohydrate polymers derived from or contained by (usually) edible plants, (ranging from DP >3 to >10) which are neither absorbed within the small intestine, nor hydrolysable by mammalian digestive enzymes in the small intestine".

The lack of fermentable fibre due to a diet poor in fibre leads to utilization of mucins from the host (Desai *et al.*, 2016) as well as protein, both endogenous as well those from the diet (Cummings and Macfarlane, 1991) which leads to negative health outcomes. Human studies have shown various unfavourable outcomes of diets which are rich in protein and poor in carbohydrates, such as reduction of SCFA production, particularly butyrate (Duncan *et al.*, 2007) along with the increase in the production of toxic compounds such as indoles, sulphides, amines and branched chain fatty acids which may lead to increased risk of chronic illnesses (Windey, de Preter and Verbeke, 2012). Epidemiological evidence suggests that low dietary fibre diets, which are rich in salt, sugar and fats can lead to conditions such as obesity (Feskens, Sluik and Du, 2014), cancer (Chajès and Romieu, 2014), diabetes mellitus (Bailén *et*

al., 2020) and cardiovascular issues (Kochar, Gaziano and Djoussé, 2011; Buttriss and Stokes, 2008).

Figure 2-4 compares how a low fibre and high fibre diet leads to different outcomes in the gut.

Fermentation can be considered as one of the primary functions of the colon or large intestine where carbohydrates are broken down by anaerobic bacteria into SCFAs mainly as acetate, propionate and butyrate (Figure 2-5), gas and other metabolic products like organic acids such as lactate and formate (Table 2-2). Acetate, propionate and butyrate comprise 90-95% of total SCFA but lesser amounts of hexanoate, valerate and branched chain amino acids (BCFA) such as isovalerate and isobutyrate (Mortensen and Clausen, 1996) are found. BCFA are mainly produced during breakdown of protein. In addition, protein fermentation also produces toxic metabolites such as ammonia, thiols and indoles (Macfarlane and Macfarlane, 2012). The nitrogenous substrates involved in fermentation, their fermentation end products and physiological effects of end products, are depicted in Figure 2-6. Most of the SCFAs are absorbed by colonocytes and metabolized by different tissues. Butyrate acts as the main source of energy for colonocytes. Only 5-10% of SCFA will be excreted along with faeces (Wu *et al.*, 2019).

Due to the numerous benefits of SCFAs, there is interest in studying the changes in microorganism profiles, extents, and rates of fermentation of different dietary fibres using various methods; *in vivo*, cell models, *ex vivo*, animal, and human studies. Human and animal trials provide the advantage of complex physiological relevance, however they can be difficult to conduct due to ethical and financial reasons. *In vitro* methods provide the advantages of being less complex, fewer ethical issues, the possibility of sampling over extended time periods, more control and the flexibility to study specific substrates and the metabolites that are produced during fermentation. However, *in vitro* methods might not effectively estimate the interactions between the gut of the host and resident microflora (Jonathan *et al.*, 2012).

Yet, *in vitro* fermentation is a great tool for initial assessments of fermentation characteristics of various substrates which maybe then be tested *in vivo.* In an *in vitro* fermentation study using pig faecal inoculum it was found that soluble arabinoxylans from wheat flour and beta glucans from barley of high, medium

and low viscosities showed similar gas profiles and fermentation end products in spite of the differences in their structure as well as viscosities. However, insoluble arabinoxylan fermented much more slowly while insoluble bacterial cellulose showed very little fermentability (Williams *et al.*, 2011). In another *in vitro* study purified arabinoxylans and mixed linkage glucans, as well as their mixture in similar proportions to wheat cell walls, were compared to actual wheat cell walls. It was found that the intact wheat cell walls fermented much slower in comparison the individual polysaccharides as well as the mix, with slower gas and SCFA production. This shows that not only the composition of polysaccharides in a substrate but also the architecture of their cell walls leads to a difference in their fermentability (Lu *et al.*, 2020). In another attempt to study the same substrates by Lu *et al.*, (2021) it was found that in the *in vitro* fermentation of arabinoxylan, mixed linkage glucan, and wheat cell walls the microbial population was similar at the genus level although these substrates produced different amounts of SCFA. Bacterial cellulose however in comparison showed a difference in the microbial population (Figure 2-7). During a study where the fermentability of bread, tortilla and noodles produced from wheat flour with different amylose contents was determined *by in vitro* fermentation, in opposition to the initial hypothesis, it was found that the form of food had a greater impact on the gas kinetics, SCFA production and the shifts in the microbial population compared to the amylose content (Bui *et al.*, 2021).

Factors affecting fermentation

Various attributes of dietary fibres affect the fermentation rate in the colon, SCFA profile produced, and shifts in bacterial populations (Gidley, 2013). These attributes include molecular sizes, arrangements, linkage compositions of polysaccharides, monosaccharide composition and arrangement of sugars in the polysaccharide chains (Hamaker and Tuncil, 2014; Shi *et al.*, 2017) (Figure 2-8). The arrangement of various polysaccharides in supramolecular matrices such as plant cell walls also have an effect on their functionality in the gut and on their prebiotic potential (Grundy *et al.*, 2016). Soluble and insoluble is a major classification used for dietary fibres. However, their solubility does not ensure fermentability since soluble fibres display a range of fermentabilities, although many display prebiotic effects for *Lactobacillus* and *Bifidobacterium* (Flint and Bayer, 2008). Soluble low molecular weight carbohydrates, like

oligosaccharides such as galactooligosaccharides, lactulose and fructooligosaccharides are used as prebiotics as they do not increase the viscosity of the medium but they are rapidly and completely fermented in animal models like mice (Gibson *et al.*, 1995; Cani *et al.*, 2007; Gibson, 2004; Gibson *et al.*, 2004). The β-fructofuranoside enzyme possessed by *Bifidobacteria* makes fructooligosaccharides easily utilizable by them (Imamura, Hisamitsu and Kobashi, 1994) . Mixed linkage β-glucans are also soluble polysaccharides mainly from cereal grains (Kumar *et al.*, 2012) which are well fermented by the microorganisms in the gut (Williams *et al.*, 2011). Pectin, which is a viscous soluble polysaccharide, is well fermented in *in vitro* fermentation (Bauer *et al.*, 2010). Soluble arabinoxylans are found in various grains like wheat and as heteroxylans in *Plantago* (Kumar *et al.*, 2012). Arabinoxylan from wheat is very viscous and is well fermented during *in vitro* fermentation using pig inoculum (Williams *et al.*, 2019). However, unlike oligosaccharides that are completely fermented, some polysaccharides like beta glucan, inulin and arabinoxylan are partially fermented at a level of only about 10% (Williams *et al.*, 2011).

Species across the *Plantago* genus, typified by the common fibre psyllium from *Plantago ovata*, contain heteroxylans, which are considered to have a high water affinity and are very viscous (Phan *et al.*, 2020; Cowley and Burton, 2021; Cowley *et al.*, 2020; Cowley, 2020; Cowley, O'Donovan and Burton, 2021). However in spite of its high solubility psyllium from *P. ovata* is very poorly fermented (Marteau *et al.*, 1994; Marlett and Fischer, 2002; Marlett and Fischer, 2003). Similarly in the category of insoluble fibre there are both fermentable and non-fermentable materials. During an *in vitro* fermentation of the soluble and insoluble components of refined flour using pig faecal inoculum it was found that they displayed similar fermentation behaviour (Comino, Williams and Gidley, 2018). Resistant starch is an insoluble dietary fibre that is slowly fermented (Warren *et al.*, 2018). In an *in vitro* fermentation study it was found that insoluble fibres in the vascular tissues are resistant to fermentation (Low *et al.*, 2015). Therefore fermentation performance of dietary fibre may not merely be classified based on their solubility (Gidley, 2013).

The chemical structure of polysaccharides is considered as a rate limiting step in fermentation. Ferulic acid crosslinked arabinoxylans are fermented slower than non-crosslinked arabinoxylans although both produced the same amount of SCFA at the end. The crosslinking increased the complexity and viscosity

of the ferulic acid crosslinked arabinoxylan. The gelled nature reduced the access of the xylanolytic enzymes and decreased their activity (Hopkin *et al.*, 2003). Side chains also affect the fermentation of polysaccharides. Side chains prevented enzymatic hydrolysis of arabinoxylan compared to linear chains due to impeded access to the backbone (Yang *et al.*, 2013). Further, the fermentation rate is affected by linkage type (Rumpagaporn *et al.*, 2015). Mikkelsen, Gidley and Williams, (2011) observed much slower fermentation of bacterial cellulose in comparison to mixed linkage glucans. Both these substrates have a similar monosaccharide composition but with different linkages which led to differences in their fermentability. In partially-hydrolysed pectin the degree of methylation has a larger impact on the rate of fermentation than the degree of polymerization (Gulfi, Arrigoni and Amadò, 2005). The crystalline type and degree of crystallinity affected the rate of fermentation in retrograded starch (Goñi *et al.*, 2000; Plongbunjong *et al.*, 2017). Bacterial cellulose with higher crystallinity was found to be less fermentable with less SCFA production, which shows physical form is also important for fermentation (Mikkelsen, Gidley and Williams, 2011). Not only does the polysaccharide structure affect fermentation rate but it also affects the types and amounts of SCFA produced. Fermentation of aldehyde sugars such as glucose, mannose and xylose mainly produce butyrate and acetate while fermentation of ketone sugars like fructose and arabinose mainly produce propionates (Hu, Nie, Li, Fu, *et al.*, 2013). Butyrate was increased when fermenting beta glucan, inulin and guar gum (Yang *et al.*, 2013), xylan (Bach Knudsen, 2015), type 3 resistant starch (Plongbunjong *et al.*, 2017) and type 4 resistant starch (Pryde *et al.*, 2002). Arabinoxylan showed higher propionate concentrations (Yang *et al.*, 2013). During *in vitro* fermentation with human faecal inoculum, both chemical and physical structure affected the SCFA ratios (Salvador *et al.*, 1993). Table 2-4 shows fermentation rates of various polysaccharides by human faecal inoculums.

Products of fermentation and their effects

Gut microflora use various metabolic pathways and reactions to produce SCFA by fermenting dietary fibre. About 90-95% of these SCFA consists of acetate, propionate and butyrate which are usually found approximately as 60%, 25% and 15% respectively (Tan *et al.*, 2014). The SCFA produced during the gastrointestinal fermentation are absorbed quickly with only 5%-10% in the faecal excretion (Cook and

Sellin, 1998) (Figure 2-9). The SCFA are absorbed and then metabolized at three sites mainly; butyrate is used in the colonic epithelium for energy production, 50-70% of acetate is absorbed by the liver and butyrate remaining after colonic metabolization along with propionate is also used for gluconeogenesis in the liver. The third major site is muscle cells where energy is produced by oxidizing the remaining acetate (Hijova and Chmelarova, 2007). About 70% of the requirement of calories by herbivorous ruminants as well as 10% of the energy requirement of omnivores, including humans, comes from SCFA (Bergman, 1990) (Table 2-3.). Functions of SCFAs in the colon include; being the main energy source for colonocytes, inhibition of pathogenic microorganisms, maintenance of the integrity of the gut barrier, improvement of mineral absorption, and reduction of pH (Topping and Clifton, 2001). Lower pH values in the presence of SCFA have been shown to kill pathogens such as *E. coli* and *Salmonella* (Cherrington *et al.*, 1991). Propionate is used in the suppression of cholesterol synthesis and gluconeogenesis (Al-Lahham *et al.*, 2010). In addition to the regulation of fat and cholesterol synthesis by propionate it also increases satiety and triggers secretion of glucagon thereby reducing the gain of body weight (Maljaars *et al.*, 2008; Canfora, Jocken and Blaak, 2015). SCFAs improve overall health as shown in Figure 2-9. Butyrate mainly acts as an energy source for colonocytes providing about 60-70% of their total energy needs (Alonso and Guarner, 2013). It also regulates apoptosis pathways thereby preventing cancers in the colon, improves gut barrier function, and reduces the translocation of bacteria.

Intestinal immunity is maintained by the cells of the epithelia, lymphocytes, phagocytic white blood cells, mucous, tight junctions and cytokine mediators. The epithelium of the gut is the first line of defence due to its tight junctions, mucus production and production of antimicrobials (Correa-Oliveira *et al.*, 2016). SCFA affects this intestinal barrier in different ways such as; being the main source of energy, improving the barrier function of the membrane by enhancing the protein expression in epithelial cell junctions (Chambers *et al.*, 2018), enhancing mucin production by the inhibition of histone deacetylase (HDAC) enzymes and increasing the gene expression (Kawamura *et al.*, 2009), reduction of damage to the DNA in epithelial cells by HDAC inhibition (van der Beek *et al.*, 2017) and enhancing the antimicrobial activity of macrophages (Schulthess *et al.*, 2019). SCFA are also shown to have a role in the prevention of

colorectal cancers (Nilsson *et al.*, 2003) through various actions such as; induction of apoptosis of cancer cells (Hinnebusch *et al.*, 2002), increasing autophagy of cancer cells (Donohoe *et al.*, 2011), promotion of growth of a healthy epithelium by energy provision (Wong *et al.*, 2006), and reducing tumour growth by increasing the expression of G-protein coupled receptors (GPR) 41 and 43 and induction of expression of tumour suppressive proteins (Tang *et al.*, 2011c.; Jan *et al.*, 2002) (Figure 2-10).

Psyllium

Plantago genus

There are over 250 species which belong to the Plantaginaceae family (Samuelsen, 2000), including *Plantago psyllium*, *Plantago major*, *Plantago ovata* and *Plantago coronopus* (Haddadian, *et al.*,2014), that have been used in the treatment of problems associated with the digestive system, respiratory system, reproductive system, cancer, skin infections, or can offer pain relief (Samuelsen, 2000 ,Chiang *et al.*, 2003) as part of herbal medicine for a long time.

The use of *Plantago* for the following conditions has been approved (WHO, 1999, 2007).

- Reduction of the rise in blood sugar after a meal

- Treatment of high blood cholesterol

- Reduction of risk of coronary heart disease

- Laxative (for treatment of constipation caused by pregnancy, diverticulosis, irritable bowel syndrome)

- Stool softener for haemorrhoid patients

Plantago ovata for food and health

The husk of *P. ovata* is a natural source of concentrated, partially soluble fibre which is dispersible in water (Cowley *et al.*, 2020). Both husk and seeds are well accepted bulk laxatives which are considered safe for consumption (Sola *et al.*, 2007). Psyllium, which is the outer layer milled off the mature dry seed of *P. ovata,* is safe to be added into a diabetic diet and has been shown to display glycaemic, lipid and hypertension control in experimental rat models (Galisteo *et al.*, 2005). Many of the health promoting

effects such as regulation of the bowels and reduction of cholesterol by psyllium in rats and guinea pig models have been related to its strong gelling ability (Fang, 2000; Romero *et al.*, 2002; Marlett and Fischer, 2003; Marlett and Fischer, 2002). It is considered a good source of dietary fibre for improving general colonic health and function, as shown using rat models (Alabaster, Tang and Shivapurkar, 1996; Nakamura *et al.*, 2004). Indeed the consumption of psyllium husk has been associated with an increase in short chain fatty acid (SCFA) production in the colon, especially butyrate and acetate as shown by Fernández-Bañares *et al.*, (1999) although it is only partially fermentable. The health promoting effects of psyllium are partly a result of the gelling properties and water holding capacity. *P. ovata* mucilage is rich in polysaccharides, the majority being heteroxylan (Phan et al., 2016, Tucker *et al.*, 2017), which may be used in the food industry as a hydrocolloid agent. *P. ovata* has been used in traditional medicine to relieve disorders of the gastrointestinal system (Table 2-5) and it has been used in various pharmacological studies in both human and animal models (Table 2-6).

There is limited published research on the application of psyllium mucilage as a fat replacer. A study done by Aghdaei *et al.*, (2012) replaced 50% of the oil content in mayonnaise with psyllium mucilage, producing acceptable sensory scores for colour, mouthfeel, taste, appearance and overall acceptability. However, compared to the full fat control, the mayonnaise containing psyllium mucilage had lower scores for texture which reduced its sensory quality. Thus, an improved psyllium mucilage from mutant lines could provide better texture and improve all other sensory and rheological characteristics of reduced fat products such as ice cream and mayonnaise. The use of *P. ovata* as a fat replacer may give additional advantages as it is a concentrated source of soluble dietary fibre (Sola *et al.*, 2007) providing additional health benefits including slowing the absorption of the remaining fat content in the product. In spite of being rich in soluble fibre wild type *P. ovata* is not well fermented in the gut to produce health promoting short chain fatty acids as shown by *in vivo* studies (McRorie, 2013, Kaur *et al.*, 2011, Gibson *et al.*,1990).

The use of psyllium in gluten replacement

Several studies have used psyllium husk for the replacement of gluten in gluten-free products. The earliest study by Haque and Morris, (1994) showed that psyllium enhances the loaf volume of gluten-free bread

due to its weak gel behaviour and water absorbing capacity, which stabilizes gas cells in the dough and prevents the loss of gas from the dough during proofing. Zandonadi *et al.*, (2009) produced gluten-free bread with psyllium which displayed scores of 93% and 100% for overall acceptability and taste respectively. Cappa *et al.*, (2013) states that addition of psyllium increased the physical properties of dough due to its film-like structure formed during kneading of the dough. High fibre ingredients such as psyllium enhance the crumb softness by retention of moisture and increasing the perception of crumb moistness due to its water holding capacity. Thus, it has a good anti-staling effect on gluten-free bread. Celiac patients generally have a low intake of fibre, thus addition of psyllium could improve the nutritional value of products, as psyllium is known to have a very high level of soluble dietary fibre (70%) which may help with glycaemic control issues in coeliac patients (Krupa-Kozak and Lange, 2019).

Fermentation of *Plantago* sp.

The human consumption of mucilage from *P. ovata* showed an increase in propionic and acetic acids. The majority of the mucilage reached the caecum intact in a highly polymerized form, which was rich in xylose and arabinose, and it promoted a higher faecal bulk. It was found to be quite resistant to fermentation and faecal bulking was caused by presence of the intact polysaccharide (Marteau *et al.*, 1994). Onset of gas production during fermentation was delayed for psyllium compared to wheat bran, with a lower total gas production (Gunn *et al.*, 2020). Psyllium feeding in rats increased stool bulk, faecal pH was reduced, and SCFA output increased and about 50% of the psyllium was excreted after partial fermentation (Edwards *et al.*, 1992). *In vitro* fermentation using faecal microorganisms as well as single strains is widely used for determining relationships between gut flora and polysaccharides. During the fermentation of polysaccharides these microorganisms produce short chain fatty acids and gases and reduce the pH, therefore these parameters are used for the evaluation of fermentation (Oliphant and Allen-Vercoe, 2019). Psyllium gel was fractionated into A (alkali insoluble), B (gel forming comprising 55%) and C (viscous soluble fraction <15%). Fraction A was not fermentable. B was poorly fermented and increases stool bulk and C was rapidly and almost completely fermented during a 72 h fermentation (Marlett and Fischer, 2002). *P. asiatica* fermentation increased total SCFA, propionic, acetic, and n-butyric

acids (Hu *et al.*, 2013b). Propionic acid was primarily produced from fermentation of xylose and arabinose while n-butyric acid resulted mainly from glucuronic acid and xylose. The pH was reduced. Xylosidase, arabinofuranosidase, xylosidase and glucuronosidase activity were increased. Consumption of total carbohydrate, arabinose, xylose, and glucuronic acid were 47.2%, 53.2%, 76.4% respectively and it was suggested that carbohydrate composition affects SCFA production (Hu *et al.*, 2013b). Also, feeding *P. asiatica* to mice reduced the pH in the colon and increased production of SCFA (Hu *et al.*, 2012). *Bifidobacteria* were only able to ferment psyllium after simulated digestion of psyllium which is speculated to be caused by changes that occur during gastric transit which may lead to the fractionation of psyllium making the prebiotic fraction of the mucilage available for fermentation by the *Bifidobacteria*. However, they were not able to metabolize it in undigested form (Elli *et al.*, 2008).

Based on all this data, *Plantago* polysaccharides are known to be slowly fermented and difficult to ferment both *in vitro* and *in vivo* (Gunn *et al.*, 2020; Marteau *et al.*, 1994), thus overall considered to be a low-fermenting fibre (Gunn *et al.*, 2020).Various treatments have been used to increase the fermentation of psyllium, such as ball milling, enzymatic hydrolysis, ultrasound and microwaving, which lead to reductions of viscosity or molecular weight, which thereby makes the polysaccharides more easily fermentable to produce more SCFA and perform better as a prebiotic (Hu, Nie, Li, Fu, *et al.*, 2013; Hu *et al.*, 2018.; Pollet *et al.*, 2012).

The need for improved forms of psyllium

In spite of the improvement of quality of gluten-free products by the addition of psyllium there is space for further improvements. Mancebo *et al.*, (2015) states that psyllium fibre is less commonly used in gluten replacement which is usually substituted by other hydrocolloids despite these often being much more expensive. However, novel forms of psyllium may be able to be used alone, thus making it a much more convenient and cost-effective gluten replacer in the food industry.

Increasing the psyllium content in bread led to an increase in hardness due to reduction in the specific volume. This had to be adjusted by increasing the water content of the dough (Mancebo *et al.*, 2015).

Similar observations were described by (Cappa, Lucisano and Mariotti, 2013), where there was an excessive hardness in the bread with psyllium where a sufficient amount of water was not added. Further, the study by Zandonadi *et al.*, (2009) states that although the gluten-free bread had 100% acceptance based on taste, 3.3% rejected the products due to its texture. Identifying new forms of psyllium with different mucilage composition to the wild type may be able to improve the texture of gluten-free bread compared to the wild type.

On the other hand, although wild type psyllium is a good source of soluble fibre, it is very poorly fermented *in vivo* as highlighted by McRorie, (2013) and Kaur *et al.*, (2011). Psyllium is widely used in the food industry and as a laxative so there is interest in improving both the fermentability and prebiotic activity of *Plantago* mucilage. Identification of novel forms psyllium that are better fermented than the wild type may provide additional benfits by improving the gut health through fermentation and increased production of short chain fatty acids.

Novel forms of Plantago mucilage

Various approaches have been used to create genetic variation in *P. ovata* , including selective breeding. However, improvement of psyllium quality, as well as yield, has been found to be long and difficult (Sarkar *et al.*, 2015). Mayuri and Niharika varieties were developed through mutagenesis (Lal *et al.*, 1998, 2006) but Kumar (2015) states that they did not have much increase in the yield advantage. Other than mutagenesis, induced polyploidy has also been used to produce variation in psyllium. Although these polyploidic plants displayed desirable characters such as increased number of heads, weight and volume of seeds, after several generations there was a reduction of germination rate and yield (Vala, Fougat and Jadeja, 2011; Zadoo and Farooqi, 1977). Since *P.ovata* is the most commercially important and well-studied Tucker *et al.*,(2017) produced gamma irradiated mutants of *P. ovata* and novel genes have been identified using them through genetic analysis (Tucker *et al.*, 2017; Phan *et al.*, 2016). James Cowley (Honours thesis, 2016) observed differences in *P. ovata* mutant mucilage using immunolabelling whilst Lina Herliana (PhD thesis, 2022) identified the high mucilage yielding mutant "*raya*".

Members of *Plantago* species such as *P. ovata* , *P. media, P. major, P .lanceolata, P.asiatica* have been found to be rich in bioactive compounds (Fons, Gargadennec and Rapior, 2008; Zhao *et al.*, 2014; Gonçalves and Romano, 2016; Navarrete *et al.*, 2016; Grubešić *et al.*, 2005). The major component of *Plantago* mucilage is heteroxylan (Fischer *et al.*, 2004; Samuelsen, 2000; Guo *et al.*, 2008) as shown by monosaccharide and linkage data. However, Phan *et al.*, (2016) who studied *P. ovata , P. major, P.debilis, P. coronopus, P. varia, P. cunninghamii* and *P . lanceolata* observed differences during oligosaccharide mapping that shows that the substitution of polysaccharide backbones vary between the species. Cowley, O'Donovan and Burton, (2021) studied 12 different species of *Plantago* and observed differences in mucilage yield, heteroxylan and pectin composition which may be useful in the industry. The whole seed flour from *Plantago* species was found to be rich in mannans, fats, protein and sugars. Cowley (2020) observed significant differences in the functionality of whole seed flour from different species of *Plantago* with varied rapid visco analysis (RVA) profiles and baking performance in gluten-free rice flour bread.

Several attempts have been made to fractionate *P. ovata* and then studying fraction composition and behaviour. Differences were observed in functional properties and heteroxylan substitution of these fractions (Ren *et al.*, 2020; Yu *et al.*, 2017; Zhou *et al.*, 2020; Guo *et al.*, 2008; Yu *et al.*, 2019; Yu *et al.*, 2019). According to Yu *et al.*, (2017) when in contact with water *P.ovata* produces a viscoelastic layer of mucilage around the seed which is arranged in three layers as L1 (cold water fraction), L2 (hot water fraction) and L3 (alkali soluble fraction). Although the fractions displayed similarities in the average molecular weights they differ in branching, molecular volumes and conformation. The L1 was a highly soluble fraction rich in galacturonic acids. L2 and L3 had similar compositions but L2 had a more open conformation and was more susceptible to hydrolysation by enzymes. Guo *et al.*, (2008) fractionated psyllium into three parts WE, AES 0.5 and AED 0.5. WE was water soluble, AES 0.5 was alkali extracted and water soluble while AEG 0.5 was alkali extracted and water insoluble. These fractions displayed differences in their linkage types and gelling capacities. AES 0.5 consisted of the largest fraction, and it had a strong gelling capacity while WE made a weaker gel. Hot water, cold water and intense extraction resistant fractions produced by a sequential mucilage extraction by Cowley *et al.*, (2020) showed

differences in monosaccharide ratios and arabinose to xylose rations (A/X). Ren *et al.*, (2020) extracted psyllium at different temperatures of 20, 40, 60, 80 and 100°C and then separated them into three groups: F1 = F20, F2 = F40+ F60, and F3 = F80+ F100 + dust. F1 was cold soluble, F2 was heat soluble and F3 was heat insoluble where F1 had the smallest A/X ratio and F3 the highest. Therefore, the higher temperature fractions are more heavily substituted than the lower temperature extracted fractions. F2 fractions displayed the highest gelation ability driven by hydrogen bonding between the side chains. According to Yu *et al.*, (2019) the highly branched nature of the more substituted fractions inhibit the interactions between the main chain of the polysaccharide molecules and lead to more hydrogen bonding. Therefore, differences in the hydrogen bonding, differences in the composition of the polysaccharides in the fractions, degree of branching, A/X ratios and differences in the motif all contribute to differences in the solubility, gel strength, melting and rheological properties of the various fractions. The differences in the functionality between the weak gels and the strong fractions maybe useful for novel applications in food and fibre such as altering the viscosity to different degrees and even imparting different textural properties in comparison to psyllium husk.

Conclusion

Plantago ovata mucilage has been used in the food industry as a hydrocolloid and is the only commercially produced species of *Plantago* mucilage. It has been found to be less fermentable but will provide laxative and faecal bulking benefits. At present there are no varied types of psyllium and all current applications in food and fibre are based on standard rheological, water holding and other properties of psyllium derived from the husk of *P. ovata*. The lack of variability of this material limits its applications due to its properties being unsuitable for alteration of the functionality of various products. The utility of only one material provides very little room for novelty. New sources of psyllium may provide alternatives to the common psyllium with differences in their functionality potentially tailored to produce food products with additional desirable properties based on the requirements of each product. There is a possibility that variations in functionality in foods or fermentability, and the related health benefits this endows, can be found in *P. ovata* mutants, in temperature dependent mucilage fractions of *P. ovata* or in materials extracted from other members of the *Plantago* genus, all of which are yet to be explored.

Figures

Table 2-1. Applications of hydrocolloids in food systems (Adapted from Saha and Bhattacharya, 2010)

Application	Hydrocolloid agent	Examples
Thickening agent	Xanthan	Soups, gravies, ketchup, desserts, toppings, filling, instant beverages
	Carboxymethyl cellulose	Ketchup, fruit pie fillings, gravies, salad dressings
	Methyl cellulose, hydroxypropyl methyl cellulose	Cake batters, whipped toppings, beverages, salad dressings
	Gum Arabic	Soft drinks, fruit juice-based beverages
	Guar gum, locust bean gum, tara gum	Ice cream, ketchup, fruit juice, cake batter, pudding powder
	Konjac manan	Noodles, jelly desserts
	Gum tragacanth	Salad dressings, bakery emulsions, fruit beverages, sauces
Gelling agent	Modified starch	Dairy desserts
	Agar	Bakery products, jellies
	Kappa-carrageenan	Tofu, milk shakes, pudding
	Low methoxy pectin	Jams, jellies, glazes, dairy desserts
	High methoxy pectin	Jams, jellies
	Gellan gum	Water-based flavoured jellies
	Alginate	Cold prepared bakery creams, restructured foods such as processed meat
	Methyl and hydroxypropyl methyl cellulose	Whipped toppings, beverages, salad dressings, cake batters
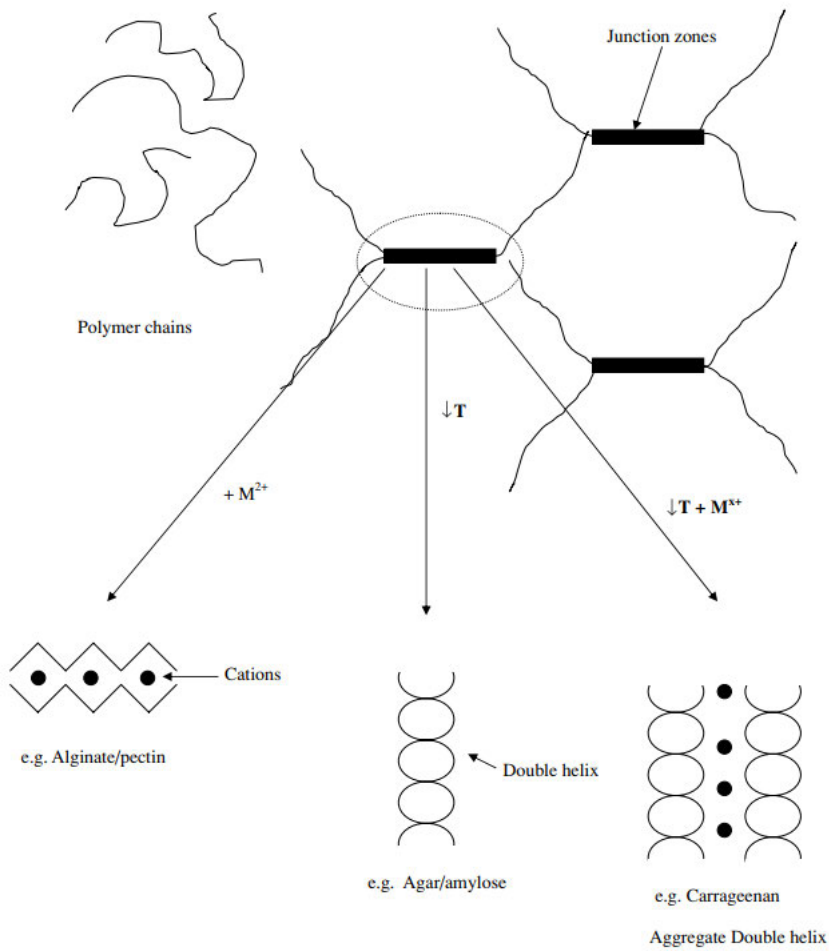
Figure 2 -1. Gelling of hydrocolloids by cross linking. T= temperature, M+ =cations (Adapted from Burey *et al.*, 2008)
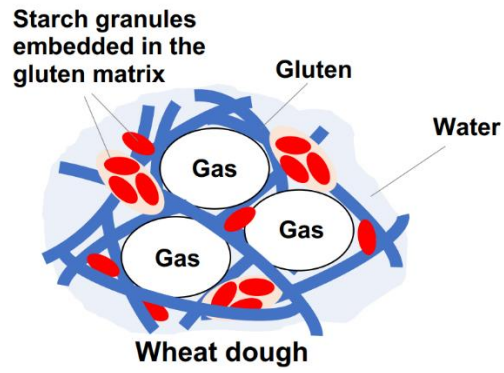
Figure 2-2. Formation of gluten network trapping air bubbles in wheat flour dough (Adapted from Yano, 2019)
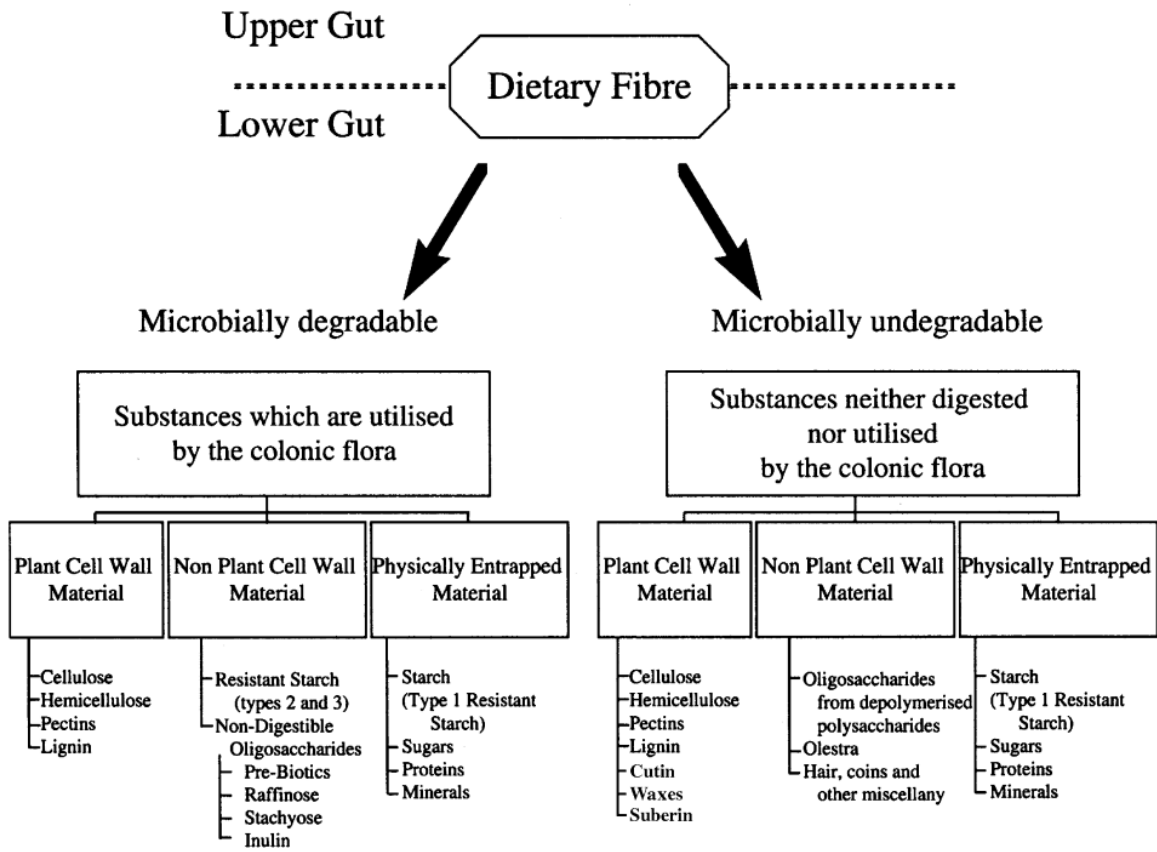
Figure 2-3. Classification of dietary fibre (Adapted from Ha, Jarvis and Mann, 2000)
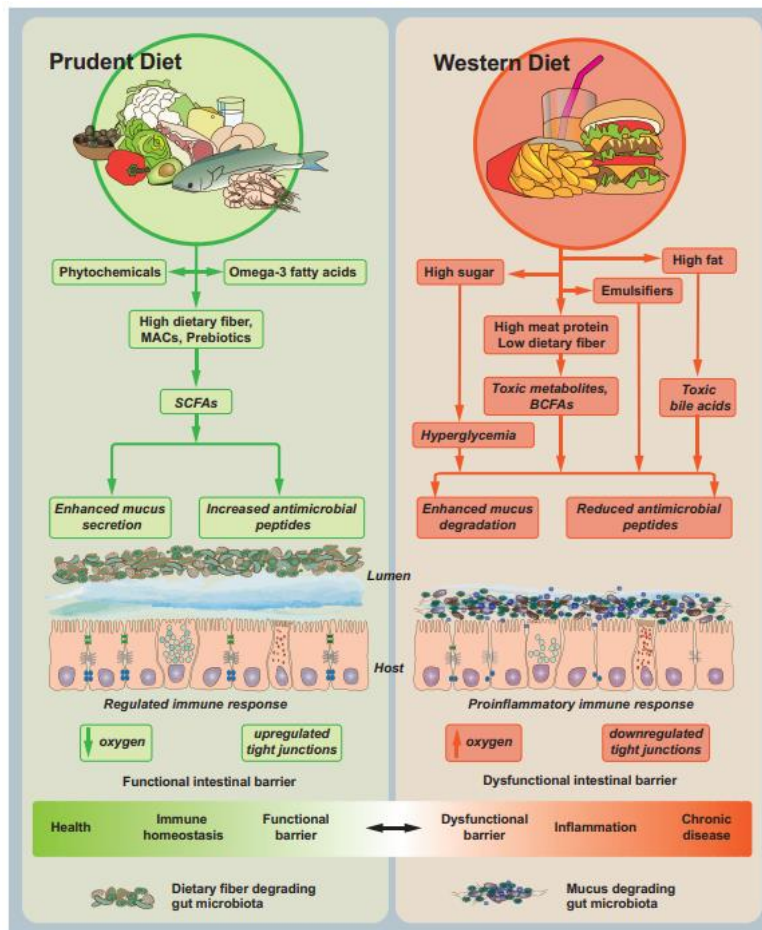
Figure 2-4. The effects of high and low fibre diets on gut health (Adapted from Makki *et al.*, 2018).
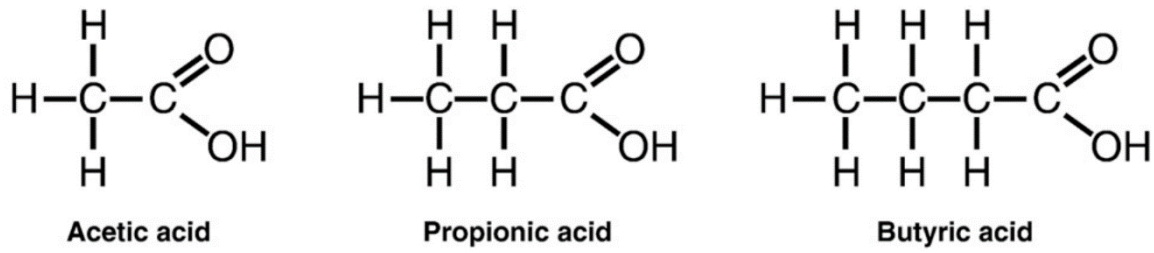
Figure 2-5. Structure of major SCFA produced by gastrointestinal fermentation (Adapted from Al-Lahham *et al.*, 2010).

Table 2-2. Examples of major genera of human gut flora and their metabolic end products (Adapted from Oliphant and Allen-Vercoe, 2019)

Phylum	Family	Genus	Substrates	End products
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	Dietary carbohydrates Human milk oligosaccharides (HMO) Mucin	Acetate Ethanol Formate Lactate
Bacteroidetes	Bacteroidaceae	*Bacteroides*	Dietary carbohydrates HMO Mucin Proteins Succinate	1,2-Propanediol Carbon dioxide Hydrogen Acetate Ethanol Formate Lactate Succinate
Firmicutes	Clostridaceae	*Clostridium* (Clostridium cluster I)	Ethanol Propionate Lactate Proteins	1,2-Propanediol Carbon dioxide Hydrogen Acetate Ethanol Formate Lactate Succinate Butyrate Valerate
	Lactobacillaceae	*Lactobacillus*	1,2-Propanediol Saccharides	Acetate Ethanol Formate Lactate Propionate Propanol
	Streptococcaceae	*Streptococcus*	Mucin Saccharides	Acetate Ethanol Formate Lactate
Proteobacteria	Enterobacteriaceae	*Escherichia*	Proteins Saccharides	1,2-Propanediol 2,3-Butanediol Carbon dioxide Hydrogen Acetate Ethanol Formate Lactate Succinate
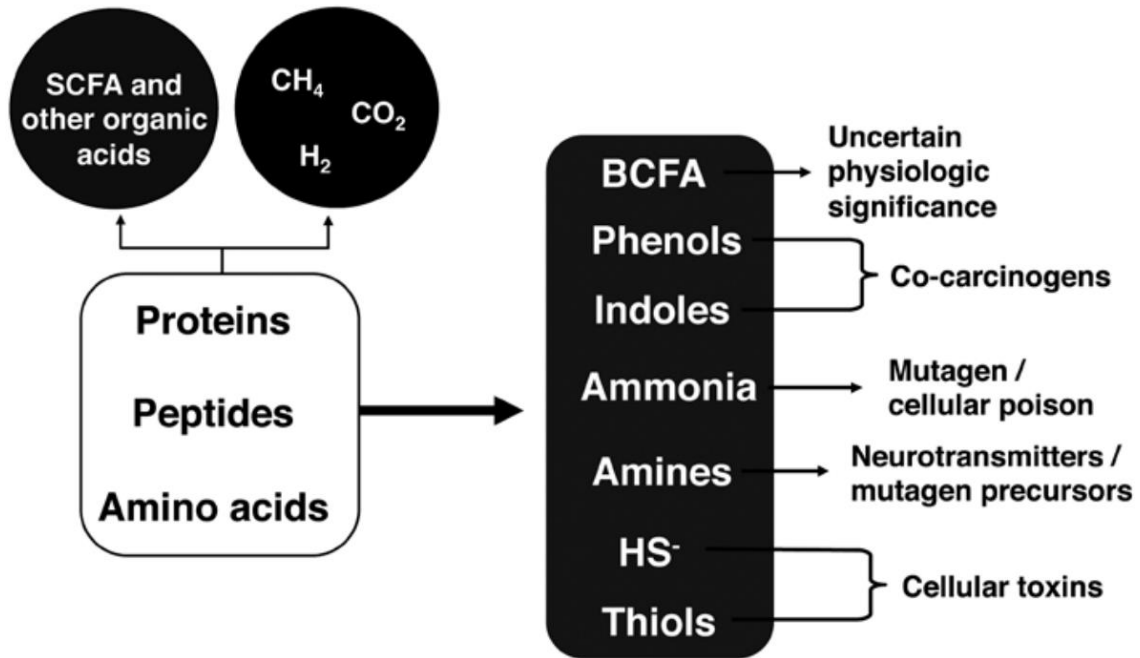
Figure 2-6. Substrates, end products of protein fermentation and their physiological effects (Adapted from Macfarlane and Macfarlane, 2012).
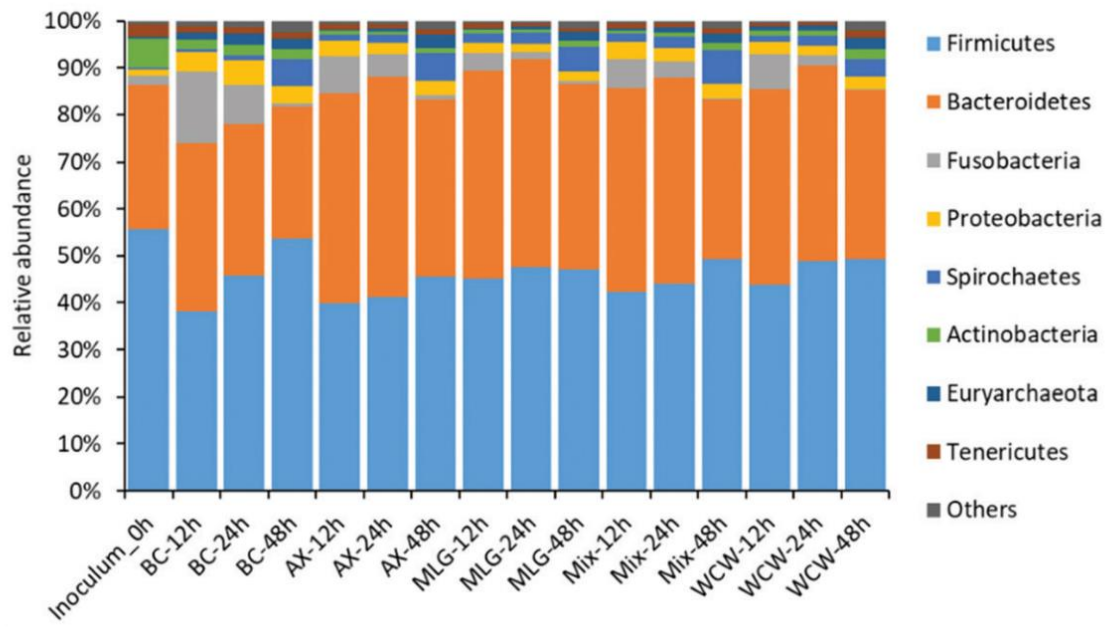
Figure 2-7. Changes in the microbial composition at phylum level during the *in vitro* fermentation of bacterial cellulose (BC), arabinoxylan (AX), a mix of arabinoxylan and mixed linkage glucans (mix) and mixed linkage glucans (MLG) using pig faecal inoculum (Adapted from Lu *et al.*, 2021)
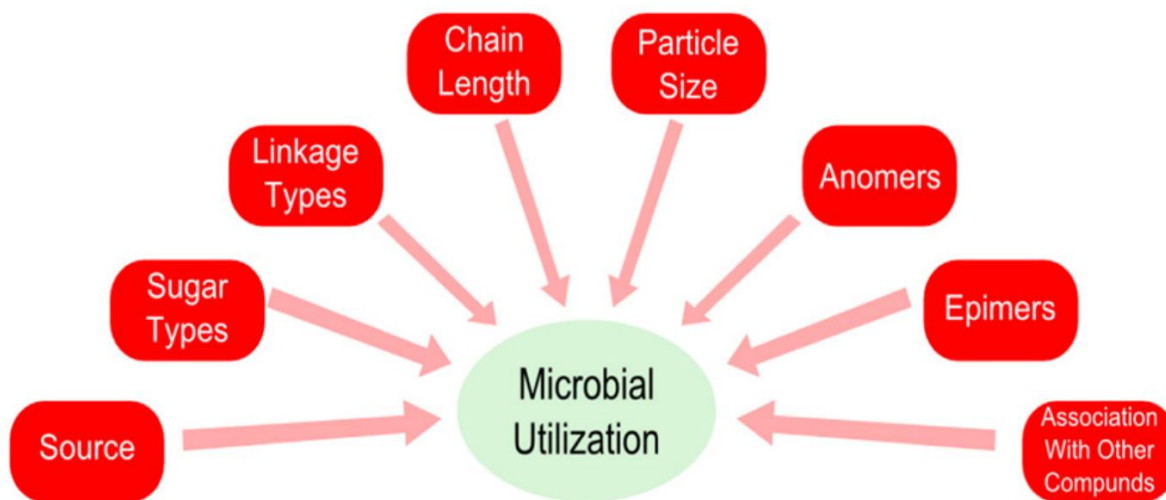
Figure 2-8. Structural variations of polysaccharides leading to differences in microbial utilization (Adapted from Hamaker and Tuncil, 2014)

Table 2-3. *In vitro* batch fermentation performance of various dietary fibres using human faecal inocula (Adapted from Wang *et al.*, 2019)

Dietary fibre	Fermentation rate	SCFA molar ratio
Psyllium husk	Limited	Acetate ↑ Propionate↑
Psyllium seed	Limited	Acetate ↑ Butyrate↑
Resistant starch type 2 (native starch granules)	Slow	Acetate ↑ Butyrate ↑
Resistant starch type 3 (retrograded starches)	Slow	Acetate ↑ Butyrate ↑
Resistant starch type 4 (chemically modified starches)	Slow	Acetate ↑ Butyrate ↑
Xyloglucan	Fast	Propionate ↑ Butyrate ↑
Inulin	Fast	Acetate ↑ Butyrate ↑
Pectin	Fast	Acetate ↑ Propionate ↑ Butyrate ↑
Guar gum	Fast	Propionate ↓ Acetate ↑ Butyrate ↑
Glucomannan	Slow	Propionate ↑ Acetate ↑ Butyrate ↓
Beta-glucan	Fast	Acetate ↑ Butyrate ↑
Cellulose	Very limited	Acetate ↑ Butyrate ↑
Fructo-oligosaccharides	Fast	Acetate ↑ Butyrate ↑
Galacto-oligosaccharides	Fast	Acetate ↑ Butyrate ↑ Propionate ↑

a)
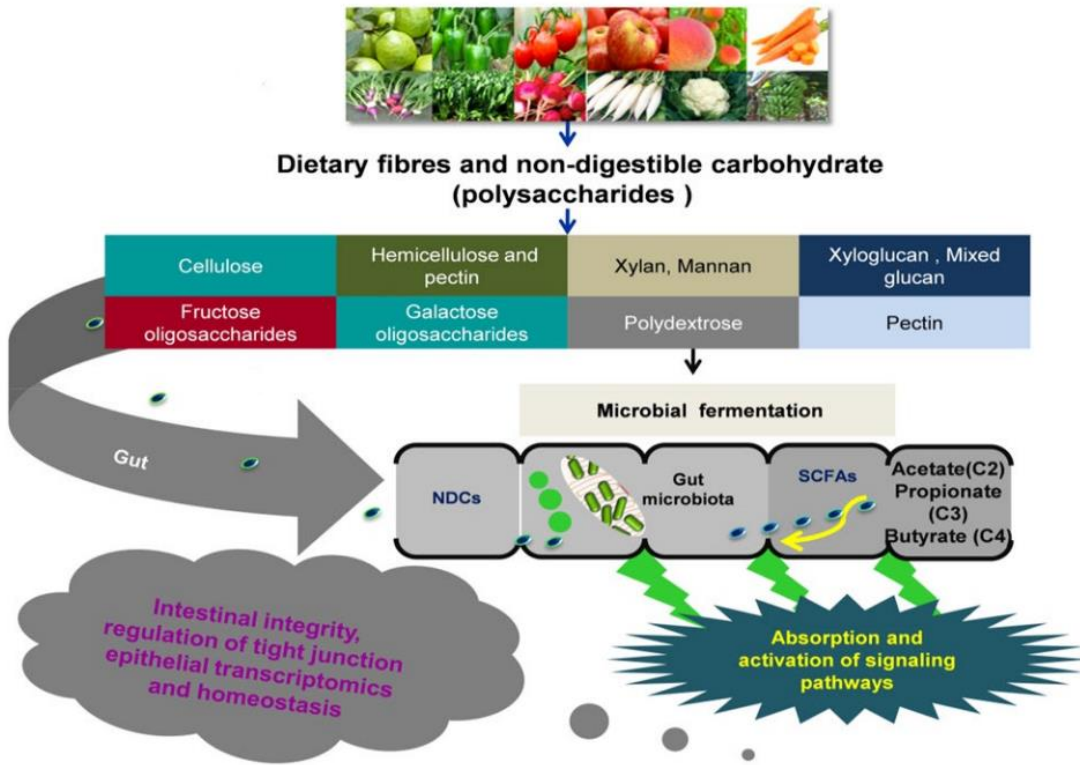
b)
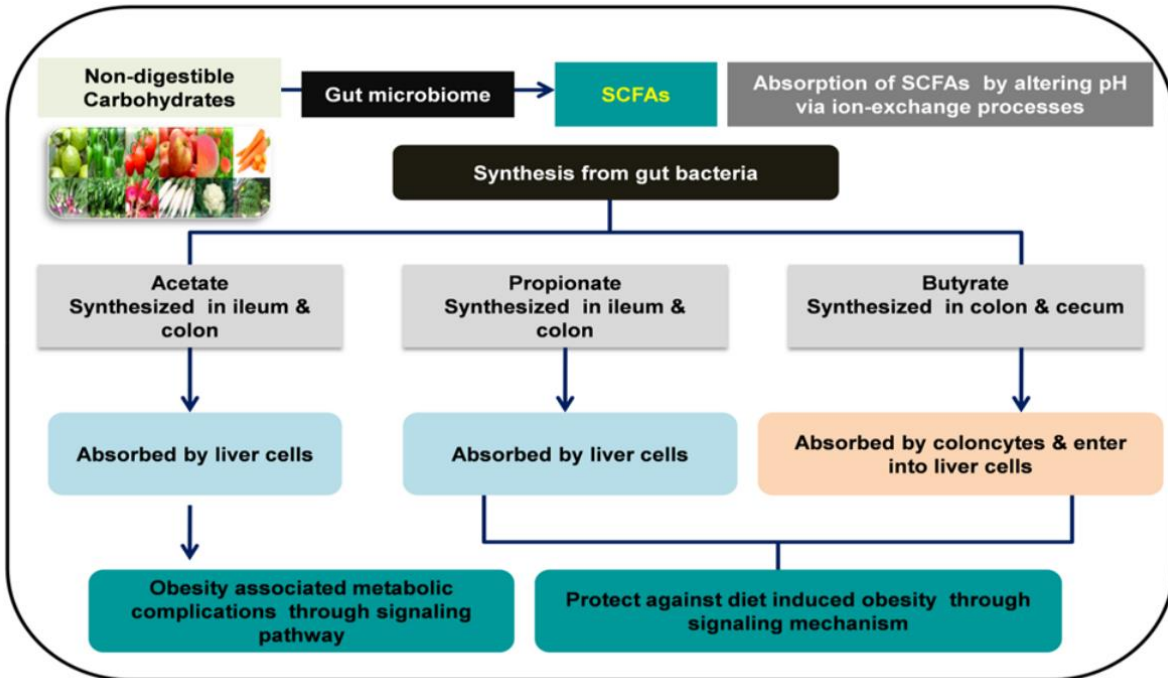
Figure 2-9. a) Summary of fermentation of common non digestible carbohydrates, fermentation products and major physiological effects b) Production of SCFA in the gut and the absorption and modulation of physiological processes of the host (Adapted from Kumar, Rani and Datt, 2020).

Table 2-4. Biological effects of major SCFA produced by gastrointestinal fermentation (Adapted from Hugenholtz *et al.*, 2013 with data sourced from Fava *et al.*, 2009; Wong *et al.*, 2006; Round and Mazmanian, 2009)

Acetate	Propionate	Butyrate	Action
↑	↑		Energy source (brain, heart, muscle)
		↑	Providing energy to colonocytes
↑	↑	↑	Reduction of gut pH
↑	↑	↑	Reduction of the absorption ammonia in gut epithelium
↑	↑	↑	Decrease in the growth of gut pathogens
		↑	Inhibition of proliferation and induction of apoptosis of cancerous cells in the gastrointestinal system
↑	↑		Lipid metabolism
		↑	Increased satiety through leptin production
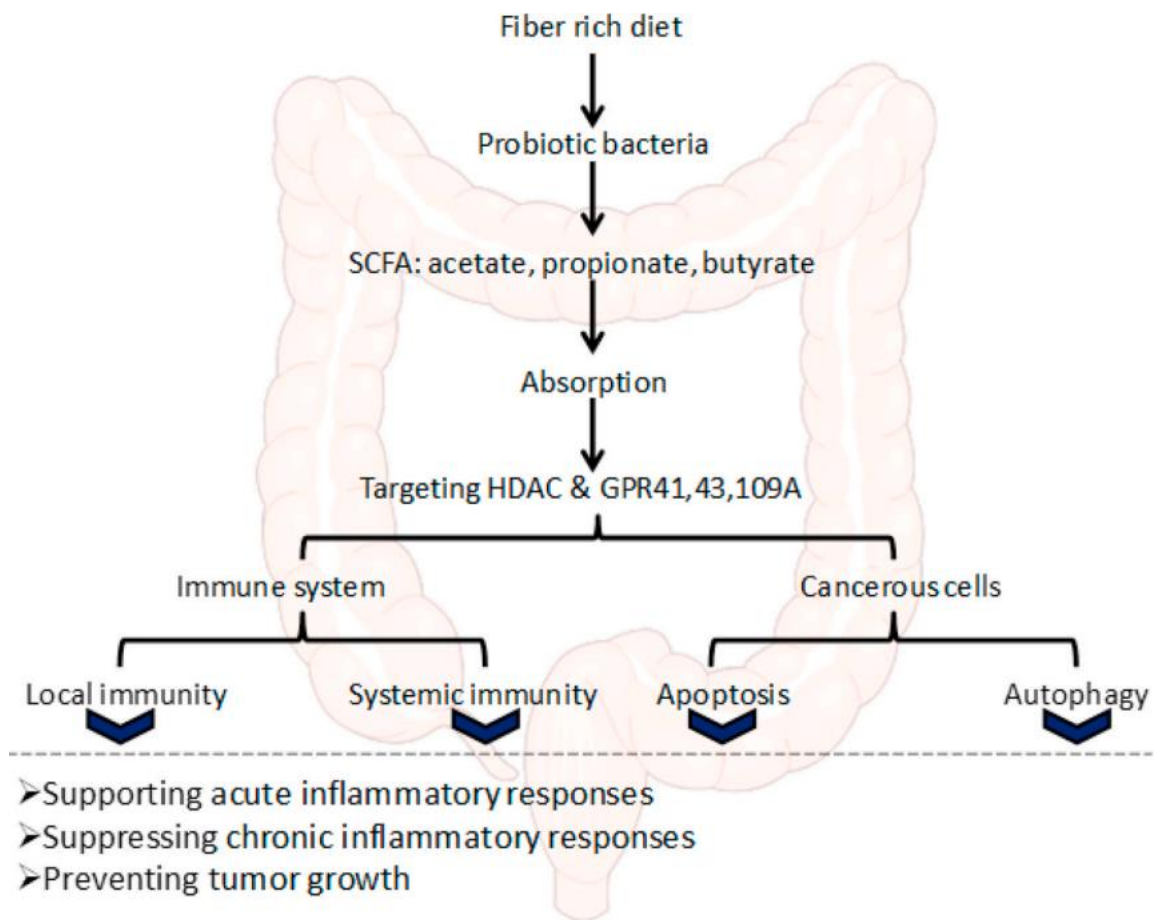
Figure 2-10. The anticarcinogenic activity of SCFA (Adapted from Fattahi, Heidari and Khosroushahi, 2020).

Table 2-5. Traditional medicinal uses of *P.ovata* in different countries   (Adapted from Ross, 2005).

Country	Method of use	Medicinal use
Thailand	Hot water extract of dry husk	Demulcent (relief from inflammation and irritation)
		Treatment of diarrhoea
Spain	Leaf infusion	Treatment of common cold
India	Decoction of dry seeds	Treatment of diarrhoea
		Demulcent
	Dry seeds	Treatment of constipation
		Relief from gastric complaints
Iran	Water extract of dry seeds	Emollient effects (soothing the skin)
		Diuretic (promote urination)
	Seeds with coconut juice	Treatment of diarrhoea
	Dry seeds	Relieving indigestion caused by bile secretion abnormalities
		Bulk laxative
		Treatment of rheumatoid arthritis
		Treatment of gout
	Seed coat	Relieving urinary tract inflammation
	Dry seed extracted with acetic acid	
	Dry seed infusion	

Table 2-6. Pharmacological activities of *P. ovata* mucilage (Adapted from Ross, 2005).

Pharmacological activity	Reference
Protective effect on intestinal mucosa	(Sahagún *et al.*, 2015)
Improvement of glycaemic control	(Gibb *et al.*, 2015)
Anti-ulcer	(Bagheri *et al.*, 2018)
Protective effect on liver	(Bagheri *et al.*, 2018)
Hypercholesteroleamic effect	(Yadav *et al.*, 2016)
Prebiotic effect	(Yadav *et al.*, 2016)
Reduction of Low-density lipoprotein cholesterol	(Solà *et al.*, 2010) (Romero *et al.*, 2002)
Anti-inflammatory activity in colon	(Rodríguez-Cabezas *et al.*, 2003)
Increased SCFA production	(Rodríguez-Cabezas *et al.*, 2003)
Obesity prevention	(Galisteo *et al.*, 2005)
Hypertension prevention	(Galisteo *et al.*, 2005)
Maintenance of remission in ulcerative colitis	(Fernández-Bañares *et al.*, 1999)

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CHAPTER 3

Plantago ovata mutants display differences in mucilage structure, composition and functionality

Abbreviations

CWE   Cold water extractable

HWE   Hot water extractable

IAE   Intense agitation extractable

WAC   Water absorption capacity

RVA   Rapid Visco Analyzer

RR   Ruthenium red

LED   Light emitting diode

RP-HPLC   Reverse phase high performance liquid chromatography

SSP   Starch swelling power

POV   *Plantago ovata*

WT   Wild type

PCR   Polymerase chain reaction

SCFA   Short chain fatty acids

DF   Dietary fibre

HCl   Hydrochloric Acid

NH_3 Ammonia

Sig.   P-value

NMR   Nuclear Magnetic Resonance

Brchpn   Branched-chain proportion (compared with total SCFA)

Brch   Total Branched-chain fatty acids

M1   *P. ovata* wild type

M2   Mutant 671-6

M3   Mutant 671-7

M4   Mutant 673-1

M5   Mutant 668-10

M(n)-W   Whole husk of M (1-5)

M(n)-F   Cold water fraction removed husk of M (1-5)

Introduction

Hydrocolloids used in the food industry are derived from various sources; algal such as carrageenan, alginic acid and agar-agar (Ruiter and Rudolph, 1997), animal like gelatin and chitin (Islam, Bhuiyan and Islam, 2017), microbial including xanthan and gellan gum (Shih, 2008) and plant such as gum arabic and inulin (Williams and Philip, 2003). Plant-derived hydrocolloids include chemically isolated gums such as guar gum, plant exudates like gum-arabic (Izydorczyk, Cui and Wang, 2005) and gums derived from seed mucilage (Phan and Burton, 2018). Seed mucilage is a polysaccharide complex released from the seed surfaces of some plant species when wetted with water (Phan and Burton, 2018). Seed mucilage is easily extractable using physical methods which offers advantages over many other common gums that require higher intensity extractions for isolation including the use of chemicals.

Psyllium gum is derived from the milled husk of *Plantago ovata* and is the most widely used seed mucilage-based hydrocolloid (Khaliq *et al.*, 2015). *P. ovata* is native to west Asia and the Mediterranean (Kumar, 2015) where it is known as isabgol or ispaghula and often called blonde psyllium commercially (Wiesner, 2013). Mature *P. ovata* seeds are milled to separate the polysaccharide-containing husk from the inner seed tissues (Cowley and Burton, 2021). The husk swells greatly upon contact with water to make a mucilage (Bahrani, 1991; Van Craeyveld *et al.*, 2009), and is used as a dietary fibre supplement to promote laxation (Marlett and Fischer, 2003; Marlett and Fischer, 2002) and therefore as a preventative treatment for diseases such as diabetes, irritable bowel syndrome, high cholesterol, obesity and constipation (Ziai *et al.*, 2005; Van Craeyveld *et al.*, 2009; Mehmood *et al.*, 2011; Wiesner, 2013; Thorburn *et al.*, 2015; Biosci *et al.*, 2015). It is increasingly being used as a thickener and stabilizer in the food industry (Milani and Maleki, 2012), as a fat substitute (Aghdaei *et al.*, 2012; Ladjevardi *et al.*, 2015), and in gluten free food as a gluten alternative (Zandonadi, Botelho and Araújo, 2009; Cappa, Lucisano and Mariotti, 2013; Mariotti *et al.*, 2009).

Apart from minor components such as cellulose and pectin, psyllium is rich in xylan-type polysaccharides with beta-1,4-linked xylose backbones and dense sidechain substitutions, and is also

known as heteroxylan (Phan *et al.*, 2016; Yu *et al.*, 2017). In spite of xylan being a common cell wall component of plants, the heteroxylan of psyllium is distinct due to its unusual linkages of beta-1,3-linked xylose and di and tri-saccharide side chains (Phan & Burton, 2018). In general, polysaccharides undergo gelation by forming backbone-backbone junction zones, however psyllium mucilage is highly branched prohibiting such interactions, and as such is thought to instead form physical gels through side chain interactions (Yu *et al.*, 2017; Yu *et al.*, 2018; Ren *et al.*, 2020a; Ren *et al.*, 2020b). The highly complex polysaccharide structure of psyllium heteroxylan confers unique technofunctional properties which makes it useful as a hydrocolloid. This unique and highly complex structure of *Plantago* heteroxylan is clearly integral to mucilage properties, both *in situ* and as a hydrocolloid ingredient. However, its unique properties mean that it is not ideal for all uses and thus sources of psyllium husk with different functionalities would potentially broaden its applicability and start building a useful hydrocolloid toolbox for manipulating food properties. It has also been reported to be poorly fermentable, providing mainly a bulking action on faeces (Marteau *et al.*, 1994; Edwards *et al*., 1992).

Plantago ovata has inherently little genetic variation and natural variation in mucilage properties to delve into is also limited. Recently, a mutant population of *P. ovata* was developed by Tucker *et al.*, (2017) using gamma irradiation to facilitate studying the genetic aspects of this complex mucilage-producing plant. Many of these mutations affected one or more of the dozens of genes involved in seed mucilage synthesis leading to a range of changes in mucilage properties, from dramatic to subtle. Work by Phan (2018) and Cowley (2016) has characterised some *P. ovata* mucilage mutants with a variety of mucilage phenotypes. Mutations may lead to structural changes in the heteroxylan as well as the composition and arrangement of other polysaccharides in psyllium which may potentially provide mucilage with altered or improved functional and fermentation properties. In this study, *P. ovata* mutant lines from the population described in Tucker *et al.* (2017) were screened for differences in mucilage structure, functionality and fermentability, which may be exploited for creating novel properties in food and as a fibre supplement.

Materials and Methods

Materials

An M3 population of *P. ovata* mutants which were mutagenized using a Co60 gamma source at 300 Gy, at the Australian Nuclear Science and Technology Organisation (ANSTO, Sydney, Australia) by Tucker et al. (Tucker *et al.*, 2017a) was used for the study. The mutant lines had been grown through to the M3 generation but no backcrossing had been performed. Therefore, each mutant line contains multiple genetic changes but in the context of this screen, dominant mutations that have persisted through to the M3 generation were sought.

Primary screening of M3 mutants

Ruthenium red screening

Mature seeds were individually placed in 96 well plates and covered with 0.2 mL 0.01% ruthenium red solution at room temperature (w/v) (ProSciTech, C075, Australia) and allowed to imbibe for 20 minutes. Screening was done by observing differences using the naked eye by placing the plates on a LED plate and comparing mutant seeds to *P.ovata* wild type. A group of 350 M3 mutant lines were observed with 12 biological replicates for each line and 25 lines were picked for further study.

Secondary screening of M3 mutants

Ruthenium red microscopy

Mature mutant *P.ovata* seeds were placed individually on microscope slides and allowed to imbibe in 0.01% ruthenium red solution at room temperature (w/v) (ProSciTech, C075, Australia) for ten minutes and observed under a Zeiss Stemi 2000-C dissecting microscope attached to an AxioCam ERc 5s camera (Phan et al., 2016) at 1X magnification. Microscopy was used to examine 25 lines selected from the primary screening with at least four biological replicates each.

Water absorption capacity (WAC)

Water absorption capacity (WAC) was determined following Cowley *et al.,* (2020) with slight modifications. Twenty seeds were weighed into 2 mL microcentrifuge tubes and 1 mL deionized water was added. The tubes were vortexed briefly to submerge the seeds, after which they were allowed to stand for 45 minutes without being disturbed. A 1000 µL pipette was used to remove the unabsorbed water. The tubes were reweighed. Five technical replicates were measured for each line. WAC was calculated using the following equation.

Water absorption capacity g/g =Initial weight of water added − weight of unabsorbed water

Initial weight of seeds

WAC of 25 lines selected from the primary screening was determined with at least three biological replicates each.

Plant growth

M3 Seeds were germinated on filter paper at room temperature and then transferred to jiffy pots for seedlings to grow until day 10. The seedlings were transplanted in coco-peat soil mixture in 2.5 litre citrus pots placed on deep plastic trays at a glasshouse in The Plant Accelerator, University of Adelaide, Australia under LED-generated light at day/night temperatures of 22 °C/18 °C from December to March. At least 12 plants were grown for each line. Watering was done indirectly by adding water to the trays holding the pots. Phenotypic data such as plant photographs, number of heads per plant and yield of seeds per plant were collected.

Microscopy of mutants

Calcofluor white imaging

Staining with 0.01% Calcofluor white (Sigma-Aldrich) was used to detect cellulose by adapting the method by Phan *et al.* (2016) by replacing ruthenium red with calcofluor white with imaging as described

for the secondary screening section. Images were obtained both at 1X and 5X magnification and processed using Zeiss® Zen software.

Whole mount immunolabelling

Distribution of various polysaccharides in the mucilage was visualized using whole mount immunolabelling using primary antibodies CCRC-M5110, CCRC-M129 (pectin) and CCRC-M110 (xylan) and the carbohydrate binding domain CBM3a (cellulose) and secondary antibody Alexa Fluor 555 goat anti-mouse IgG following Phan *et al*. (2016) and Tucker *et al*. (2017). Images were taken using a Zeiss® Axio Imager M2, attached to a Zeiss® AxioCam MR R3 camera at 5x and 10x magnifications. At least four biological replicates were imaged for each mutant line. Images were processed using ZEN 2012 software (Zeiss, Germany).

Biochemical properties of mutants

Sequential mucilage extraction

Mucilage was extracted from mutants following the protocol by Cowley *et al*., (2020). Three mucilage fractions were collected, using cold water (CWE) at 25 °C , hot water (HWE) at 65 °C plus an intense agitation extractable (IAE) fraction using a tissue disruptor-type mixer mill (Retsch MM400, Germany).The extracts were freeze dried (Labconco Freezone 6, USA) to a constant weight.

Monosaccharide analysis

Monosaccharide profiles were determined using reverse phase high performance liquid chromatography (RP-HPLC) of 1-phenyl-3-methyl-5-pyrazoline (PMP) derivatives as per Cowley *et al*. (2020). Each fraction was redissolved at 1 mg/mL in Milli-Q water and digested in acid for 3 hours following Phan *et al*. (2016). Standard curves were used to calculate concentrations of arabinose, fructose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, ribose, rhamnose and xylose (Sigma Aldrich, USA).

Food related functional properties of mutants

Starch swelling power (SSP)

Starch swelling power was determined using the method by Liu, Eskin and Cui, (2003) with modifications. Starch mucilage dispersions were made by mixing a 1000 µL volume of 1.25% rice starch solution (S7260, Sigma Aldrich) with a 1000 µL volume of 0.1% (w/v) mucilage solution in a 2 mL microcentrifuge tube with heating for 10 minutes on a thermomixer at 99 °C and 700 rpm. A starch-water suspension was included as a control. The sample was cooled on ice for 5 minutes and centrifuged at 11200 g at 5 °C for 15 minutes and the supernatant was removed. Swelling power was determined as the ratio of weight of the wet sediment in comparison to the initial weight of dry starch. Each mutant's swelling power was determined at least in triplicate. Four biological replicates with at least three technical replicates were analysed for each mutant line.

$$SSP = \frac{\text{Initial weight of starch mucilage suspension - weight of supernatant removed}}{\text{Dry weight of starch}}$$

Rapid visco analysis (RVA)

Pasting properties of rice flour-mutant husk (Rice flour, Doves Farm, United kingdom) and rice starch-mutant husk (S7260, Sigma Aldrich) mixtures were determined by Rapid Visco-Analysis using an RVA Super 4 (Newport Scientific, Australia) for standard temperature pasting (<100 °C) based on Díaz-Calderón *et al.*, (2018) with modifications. Suspensions were prepared in the RVA canister with 2.5g rice flour/rice starch and 25mg of *P.ovata* mutant husk (1% w/w) and 24 g of deionized water. The suspension was held at 25 °C for 2 min before heating to 95 °C (standard temperature) at 12 °C/min, holding at 95 °C for 2.5 min before cooling to 25 °C at 12 °C/min, with a final 2 minute holding period. During the first minute, the suspension was homogenised at 960 RPM, followed by constant stirring at 160 RPM for the rest of the test. The pasting temperature (temperature at which highest viscosity is observed), peak viscosity (highest viscosity during the run), trough viscosity (lowest viscosity during

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holding at highest temperature), breakdown viscosity (difference between peak and trough viscosity), final viscosity (viscosity at the end of run) and setback viscosity (difference between final and trough viscosity) were collected in triplicate.

Emulsification properties

The emulsification properties (Emulsion capacity=EC and Emulsion Stability=ES) of mucilage was determined by modifying the methods by (Sciarini *et al.*, 2009) as follows. For determination of EC, 1 mL mucilage suspensions of concentrations 0.1% (w/v) was mixed with 1 mL commercial canola oil (Crisco brand, Australia) in 2 mL microcentrifuge tubes (Eppendorf, Germany). The mixture was homogenized on a MM400 mixer mill (Retsch, Germany at 30 Hz for 1 minute. Then the suspensions were centrifuged (Eppendorf 5424, Germany) at 11200 RCF for 10 minutes and allowed to stand for 30 and 60 minutes. Where E_H and T_H are the height of the emulsion volume and the total height of the suspension respectively, the emulsion capacity was calculated as follows. It was assumed that the centrifuge tube had a uniform internal diameter therefore the height of the liquid column would be proportional to the volume. All measurements were made with at least six replicates.

$$EC = \left(\frac{EH}{TH}\right) 100$$

The ES at high temperature was determined by heating the emulsions on a thermomixer (Eppendorf ThermoMixer® Comfort, Germany) at 80 °C for 30 minutes followed by centrifuging (Eppendorf 5424, Germany) at 11200 RCF for 10 minutes. Where F_{EH} and I_{EH} are final emulsion height and initial emulsion height respectively, emulsion stability was calculated as follows.

$$ES = \left(\frac{FEH}{IEH}\right) 100$$

Foaming properties

The foaming properties (Foaming capacity=FC and Foam Stability=FS) of mucilage was determined by modifying the methods by (Sciarini *et al.*, 2009) as follows. For the determination of FC 2 mL suspensions of 0.1% (w/v) (1 mg/mL) were added to labelled 10 mL centrifuge tubes. The initial height

of the liquid column (H_1) was measured. The tubes were vortexed at top speed in a horizontal position with the tip end touching the vortex mixer for 1 minute. Following this the height of liquid column was measured (H_2). Foam capacity was calculated as follows. It was assumed that the centrifuge tube had a uniform internal diameter therefore the height of the liquid column would be proportional to the volume. All measurements were made of at least six replicates.

$$FC = \left(\frac{H2 - H1}{H1}\right) 100$$

The height of the liquid column was measured after 30 minutes (H3) and the foam stability was calculated as follows.

$$FC = \left(\frac{H3 - H1}{H1}\right) 100$$

In vitro fermentation of *P. ovata* mutants

Substrate Preparation

Plantago ovata husk was separated from the whole seeds by milling for 30 s in a spice grinder (BCG200, Breville, Australia), followed by stirring in a strainer to separate the husk from the seed. The husk samples from each line M1, M2, M3, M4 and M5 were placed in 50 mL centrifuge tubes with 80% (v/v) ethanol at a 1:5 ratio of husk: ethanol (w/v). The husk/ethanol mixtures were kept on a preheated magnetic stirrer and stirred at 70 °C for 30 minutes to remove any free sugars. The tubes were centrifuged at 1200xg for 5 minutes. The supernatant was discarded and the pellet was air dried. The dried pellet was ground to a fine powder using a spice grinder and used as substrate M(n)-W. A part of the husk material, with free sugars removed, was air dried followed by cold water extraction by modifying the methods by Cowley *et al.*, (2020). Husk was mixed with milliQ water at a 1:300 (w/w) ratio in beakers placed on a magnetic stirrer for 1.5 h at 25°C. The mixture was transferred into 50 mL centrifuge tubes followed by centrifugation at 13000 rpm for 2 minutes. The supernatant was very carefully removed using a 1000 μL laboratory pipette without disturbing the pellet and the dense

mucilage attached to the husk. The pellet was then freeze dried and ground into a fine powder using a spice grinder and used as substrate M(n)-F.

Dry matter of substrates

For each substrate the dry matter was determined in triplicate, by calculating the percentage of the difference of initial weight and final weight of 0.5 g of each substrate oven dried at 105 °C until a constant weight was reached.

In vitro fermentation

The *in vitro* fermentation was carried out using the methods of Williams *et al.*, (2005) as modified by Lu *et al.*, (2020). Briefly 0.2 g of substrate was accurately weighed into 60 mL serum bottles in duplicate for each time removal (0 h, 4 h, 10 h, 24 h, 48 h and five replicates each for the 72 h endpoint) for incubation with the human faecal inoculum at 37 °C. Anaerobic media was prepared using the methods described by Lowe *et al.*, (1985), and modified by Williams *et al.*, (2005). The gas volume produced and the headspace pressure was directly recorded using an AGRS (automated gas recording system-AGRS-III designed by the College of Animal Science and Technology, China Agricultural University) (Yang *et al.*, 2014) (refer to Appendix 1 for substrate details for each time removal).

Faeces were collected with approval from the Human Research Ethics Approval Committee of the University of Queensland (Approval Number 2017/HE000502), from six donors of age 18+, on an unrestricted diet, self-reported to be free from gastrointestinal illnesses with no antibiotics taken within three months of the collection date. The faeces were pooled and homogenized in a ratio of 1:5 with a 0.9% saline solution, pre-warmed to 37 °C, followed by filtration using four layers of muslin cloth. Each serum bottle was injected with 2.5 mL of inoculum under a steady stream of CO_2 to maintain anaerobic conditions and incubated at 37 °C. At each removal time bottles taken from the incubator were plunged into an ice-water bath for 20 minutes to retard to microbial activity, after which post-fermentation sampling was carried out.

Post fermentation analyses

Timed removals were made at 4 h, 10 h, 24 h, 48 h and 72 h. At each time point (3 x 1 mL) of the liquid at the top of the bottle was sampled for SCFA and NH_3 determination. SCFA samples were stabilized using a protein precipitant and NH_3 samples were stabilized using 0.2 M HCl followed by freezing at -20 °C until analysis. The remaining content of bottles were manually swirled and transferred to a 50 mL tube for pH measurement.

NH_3 analysis

Samples collected for NH_3 analysis post-fermentation were stabilized using 0.2 M HCl, leading to formation of NH_4^+ ions. The quantification was carried out following a modified protocol of Baethgen and Alley as reported by Yao *et al.,* (2022). The absorbance reading was recorded at 650 nm using a UV/visible spectrophotometer (Shimadzu UV-1800, Japan) following the reaction of ammonium with sodium nitroprusside and sodium salicylate in a weak alkaline buffer.

SCFA analysis

Samples collected for SCFA analysis were stabilized using a protein precipitant solution. The sample was centrifuged to remove any particles. From the supernatant 700 μL was added to 100 μL, (0.2 mg/mL) Trimethylsilyl propanoic acid sodium salt (TSP) solution in D_2O, present as an internal standard, followed by performing $1 H$ NMR on a Bruker 700 MHz spectrometer with a 5 mm TCI cryoprobe operating at 298 K. ^1H NMR spectra were recorded using a standard excitation sculpting pulse program with an 11 s relaxation delay for 64 scans. Seven SCFAs including propionic, n-valeric, n-butyrate, acetate, iso-valeric and iso-butyric were identified and quantified according to published values (Yao *et al.*, 2022). Data was processed using TopSpin 3.6.3 software.

Statistical analysis

Statistical differences in functional properties between mutant lines, the control and WT were determined by independent Student's *t*-test in Microsoft Excel.

Pearson correlation analyses were carried out for the mean values of fermentation parameters; acetate, propionate, butyrate, branched SCFA, NH_3, pH at 72 h and mean monosaccharide composition values using SPSS 27 (IBM SPSS Statistics, 2021) software. A P-value of less than 0.05 was regarded as statistically significant.

Results and Discussion

Screening techniques highlight differences in seed mucilage of *P. ovata* mutants

For the primary screening of *P. ovata* mutant seeds for mucilage differences, a simple technique of imbibing whole mature seeds in 0.01% ruthenium red stain was used. Once the seeds were placed in the 96 well PCR plate and the stain added, the extrusion of mucilage began immediately, staining the mucilage a pink colour. Twelve replicates were assessed using the naked eye for each of the 350 mutant lines screened and observations were recorded for differences in mucilage colour intensity, size of extruded mucilage and number of replicates displaying the same phenotype as a fraction n/12 (data not shown). Following this preliminary screening 25 mutant lines showing prominent visual differences in RR screening were selected for a secondary screening. Ruthenium red is a stereoselective stain that binds to acidic polysaccharides in the mucilage, allowing for observation of detailed mucilage structures and subsequent diagnostic screening of mucilage mutants (Tucker *et al.*, 2017b; Voiniciuc *et al.*, 2015; Cowley *et al.*, 2020). Comparing mutants using the naked eye against *P. ovata* wild type provides a quicker way to screen mucilage differences of many mutant lines against WT within a shorter period.

The 25 mutant lines selected for further investigation from the primary screening were put through a secondary screening process (Table 3-1). They were observed under the light microscope after imbibing with 0.01% RR stain to provide a clear view of the structure of the mucilage (Table 3-1B). The 25 mutants chosen for further analysis displayed differences in mucilage extrusion patterns to WT, ranging from subtle differences like 665-10 to others displaying more significant differences such as 671-10 (Table 3-1B). Between the 25 mutant lines it was observed that some lines were more intensely stained pink, like 870-3 and 871-8 while others appeared less intensely stained compared to WT like 841-8 and 681-4 (Table 3-1B). Differences were observed in area of mucilage extruded with some extruding a larger envelope such as 870-3 and 839-2 and others a smaller area like 881-2 and 671-11 (Table 3-1B). For some lines only a few seeds n/12 displayed a different phenotype to WT, like 836-2, while others displayed 12/12 for a different mucilage phenotype from WT, including 681-4 and 671-11 (Table 3-1A).

The WT seed was observed to have a dark red brown colour, with a cloud-like mucilage extruded around the seed (Table 3-1B WT commercial). The innermost layer L1 was stained a dark pink colour. This was followed by a colourless layer L2 and then a pink-stained outer layer L3. The interface between L2 and L3 was stained an intense pink. The L1 was the thinnest and L3 was the thickest (Table 3-1B). Certain mutants displayed no such layers but instead had only a tight pink ring around the seed such as 881-2 and 881-7 (Table 3-1B). Others showed staining in similar layers to WT but with characteristic patterns within each layer of extruded mucilage such as 642-6, 870-3 and 871-8 (Table 3-1B). Some displayed weak mucilage which easily dispersed and became disorganized with a simple nudge of the PCR plate like 836-10 (Table 3-1B). Cowley (Honours thesis, 2016) also observed differences in the layers, patterns and compactness of the mucilage of different lines of *P. ovata* mutants produced by Tucker *et al.*, (2017). Similar to the observations made of the *P.ovata* mutants in this study, Cowley *et al.*, (2020), also observed differences in the size of extrusion of mucilage envelope and the pattern and intensity of staining among different mutant lines produced by Tucker *et al.*, (2017) which were different from that of WT. The results of the ruthenium red imagining suggests that gamma irradiation of *P. ovata* has given rise to changes in the arrangement of acidic polysaccharides in the mucilage as layers around the seed. The differences in the intensity could be due to the variation of the amount of acidic polysaccharides in the mucilage of each mutant. These differences were explored further in the 12 mutant lines selected from the secondary screening process.

Water absorption capacity (WAC) was determined by comparing weights of dry seeds to weights when they were completely swollen. While some mutants displayed very similar WAC to WT, like 665-10, some displayed significantly higher values, like 836-10, or lower WAC such as 671-10 (Table 3-1C). WAC indicates the amount of water that can be held per unit of mutant *P. ovata* seed. The differences among mutants suggests that mutation has led to structural changes in the polysaccharides which has led to a variation of the affinity of the polysaccharides in the mucilage to water and its binding ability, a trait that was also explored further.

Based on results of RR microscopy (Table 3-1) and WAC, 12 mutant lines which showed largest differences from WT were selected for further investigation. Two pairs of sister lines 671-6 and 671-7 and 841-8 and 841-10 which showed differences in phenotype were among the 12 selected lines. As large amounts of seed were required for further studies, the M3 mutant seeds were grown to produce the M4 generation and this provided an opportunity to phenotype the plants.

Growth phenotypes of selected *P.ovata* mutants

Plantago ovata WT and the 12 selected mutant lines were grown in a glasshouse under identical conditions. Some differences were observed in plant phenotypes (Figure 3-1), with the most significant plant phenotypes described here. Mutant 671-6 displayed higher vegetative growth compared to WT with broader, longer, and more numerous leaves. However, a smaller number of flowering heads were produced by this line. Mutant 671-7 had smaller plants with very few heads (Figure 3-1). While other plants had heads oriented upwards the heads of these plants drooped down. All other lines flowered at a similar time to WT but 671-7 reached maturity very slowly and bloomed two weeks later. Plants of lines 681-3, 842-11 and 836-10 were more compact (Figure 3-1) and for 681-3, two out of 19 plants grown were male sterile, producing no anthers. Additional growth phenotypes were seen where 841-10 had few heads (Figure 3-1) and 673-1 had very bright green leaves which were broader than all other plants (Figure 3-1).

Mutants displayed differences in average number of heads per plant, length of head and yield of seeds as shown in Figure 3-2. Mutant 841-8 and WT had similar numbers of heads (Figure 3-2A), but all other 11 lines had significantly lower number of heads ($p<0.05$) per plant, with 671-6, 671-7, and 841-10 being the lowest (Figure 3-2A).

Line 841-10 showed a large variation in the lengths of heads on the same plant although the mean length of heads was not significantly different ($P>0.05$) from WT (Figure 3-2B). All mutants except 841-10 and 870-3 had significant differences in the length of heads compared to WT ($P<0.05$) (Figure 3-B).

Particularly lines 671-6, 836-10 and 841-8, which had much shorter heads compared to WT (Figure 3-2B).

All plants displayed significantly lower yield compared to WT ($P<0.05$) (Figure 3-2C). Mutants 671-6 and 671-7 had the lowest compared to WT (Figure 3-2C). Not only did the mutants have low yield per plant compared to WT, but they also had lower seed yield per head (Figure 3-2D) except for 870-3 and 836-10. While WT had about 105 mg of seeds per head on average, the yield per head of 671-6 and 671-7 was less than half of this (Figure 3-2C).

Phan (PhD thesis, 2018) also observed differences in plant growth for the mutant *hik.* These plants displayed reduction in vegetative growth with stunting, reduced number and size of seed heads, low yield, low viability of seeds and even male sterility such as seen for 681-3. Variation in plant growth characteristic of the *P.ovata* mutants in this study suggests that gamma irradiation has not only affected genes that control the characteristics of mucilage from seeds but has induced pleiotropic effects by affecting other genes in the same background. Stearns, (2010) defines pleiotropy as "the phenomenon in which a single locus affects two or more apparently unrelated phenotypic traits and is often identified as a single mutation that affects two or more wild-type traits." In some cases, gamma irradiation can cause loss of large chromosomal sections and there is usually a background level of lethality (Vizir and Mulligan, 1999). Here, lines were selected on the basis of dominant mutations which have persisted through to the M3 and M4 generations. Therefore, even if a mutant displays desirable mucilage characteristics the reduction of yield and plant viability might have a major negative impact on downstream use of such material directly for commercial purposes. This could be rectified using multiple rounds of backcrossing to the WT followed by selection to remove deleterious background effects and make selected lines economically viable. This is a routine approach in any mutation breeding strategy (Begna, 2022) and would be one of the next steps following the work presented in this thesis to identify promising lines for further development.

Microscopy reveals differences in seed mucilage distribution in *P.ovata* mutants

During the screening process using ruthenium red, differences in seed mucilage architecture between the mutants and WT were detected (Table 3-1). To visualise the arrangement of mucilage around the *P. ovata* seeds and the distribution of key mucilage components, light microscopy, fluorescent microscopy and immunolabelling were used (Figure 3-3). Only six of the mutants displayed significant visible differences from WT and so they were selected for further investigation.

A solution of 0.01% ruthenium red was used to imbibe whole seeds that were then observed under the light microscope. RR binds to acidic polysaccharides like pectin and stains a bright pink colour (Figure 3-3, ruthenium red). In *P.ovata* mutants and WT the mucilage extruded in a cloud-like pattern around the seed. For the *P.ovata* WT immediately next to the seed coat there was a lightly stained layer L1, followed by a colourless area L2 and then a pink stained area wider than other layers, L3. In L3, there were areas of darker and lighter shades of pink. The interface of L2 and L3 stained intensely pink, visible as a scalloped dark pink border between L2 and L3 (Figure 3-3, WT-ruthenium red). In 671-6 this layered structure observed in WT was disorganized. Although there was still a colourless L2 layer it was narrower, and no scalloped dark line was present. L3 was very patchy with specks of dark pink all over. Parts of the mucilage of outer layer L3 appeared to be loose and swirling out into the excess stain around the seed, (Figure 3-3, 671-6-ruthenium red), and a gentle nudge of the microscope slide was sufficient to disperse the mucilage and destroy all structure. Mutant line 671-7 did have a clear L2 layer, but the scalloped line was not very prominent. However, the L3 layer was highly stained in a few pink "ruffle-like" layers (Figure 3-3, 671-7-ruthenium red). For line 681-3 a wide colourless L2 was observed with a very prominent scalloped interface. However, the L3 layer was narrower and appeared condensed and disorganized (Figure 3-3, 681-30-ruthenium red). Line 669-9 displayed a wide L2 layer followed by "ruffle like" layers in L3 (Figure 3-3, 669-90-ruthenium red), whereas 673-1 had an unstained L2 layer and a scalloped interface (Figure 3-3, 673-1-ruthenium red). However, instead of a tight pink line the pink colour seemed to be intense at the edge of the scalloping, gradually fading from pink to colourless closer to the seed. The pattern of labelling in L3 was also different from the WT. In

668-10 there was a wide L2 layer, however no scalloped interface between L2 and L3 was present and L3 appeared as a condensed narrow ring which was stained darker with rough edges (Figure 3-3, 668-10-ruthenium red).

Similar to this study, three layers, a cloud like layer, a middle layer and an inner unstained layer had been previously observed in the ruthenium red staining of WT *P. ovata* with the middle layer containing hexagonal platelets (Tucker *et al.*, 2017b). Phan (PhD thesis, 2016) also observed these three layers and differences in the expansion rate, mucilage arrangement and staining of the mutant 'hik'. Cowley *et al.*, (2020) also showed differences in the ruthenium red staining patterns of *P. ovata* mutants. Phan *et al.*, (2016) studied the ruthenium red staining of diverse *Plantago* species and stated that differences in the staining indicated altered mucilage composition. In this study there were differences observed in the intensity of staining, expansion of the mucilage, arrangement of layers, as well as characteristic patterns of staining which suggests that not only is there is a difference in the amount of acid polysaccharides but there is also a difference in their arrangement as layers around the seed. It is possible that the acidic polysaccharides in the mutants are also structurally different from that of the WT.

Calcofluor white (CW) binds to beta-glucans that contain both 1;3 and 1;4 linkages, such as xyloglucan, callose and cellulose (Harrington and Hageage, 2003). It produces a bluish-white fluorescence in the presence of violet or ultra violet light (Harrington and Hageage, 2003). Whole seeds stained with CW were observed under the fluorescent microscope (Figure 3-3, calcofluor white). In WT the staining began just at the edge of the seed, however the intensity was lower closer to the seed with the highest intensity observed in the middle layer and then decreasing in the outermost layer (Figure 3-3, calcofluor white). The staining in the middle layer was more regular and sharper than the other layers and showed a highly organized "scale-like" pattern (Figure 3-3, WT-calcofluor white). For line 671-6 the inner and outer layers were lightly stained, and the middle was much brighter. However, the scale-like pattern was not observed. Mucilage appeared highly disorganized and patchy staining was observed (Figure 3-3, 671-6-calcofluor white). For line 671-7 no regular pattern was observed, and staining was very light (Figure 3-3, 671-7-calcofluor white) as also for 681-3 (Figure 3-3, 681-3-calcofluor white), however, the

edges were organized in a manner similar to petals around the centre of a sunflower. The individual petal-like areas had a patchy staining pattern (Figure 3-3, 681-3-calcofluor white). For line 669-9 (Figure 3-3, 669-9-calcofluor white), this sunflower like pattern was observed and each petal had scale-like areas inside. For line 673-1 (Figure 3-3, 673-1-calcofluor white), the sunflower-like pattern of mucilage edges was highly prominent, with a scale-like pattern inside which was intensely stained and organized very regularly. Clear unstained areas were observed between the large sunflower petal-like stained areas. Line 668-10 (Figure 3-3, 668-10-calcofluor white) also displayed scale-like patterns but with much smaller scales and towards the mucilage edge it became more disorganized and lightly stained.

CBM3a is a probe that recognizes several polysaccharides including xyloglucan and particularly crystalline domains on cellulose (Phan *et al.*, 2016). CBM3a was used to label cellulose using whole mount immunolabelling (Figure 3-3, CBM3a). In WT the area close to the seed was less intensely labelled followed by fine rays directed outwards from the seed. The outer area was more organized and appeared as a clear line made of "test tube-like" structures arranged contiguously (Figure 3-3, WT-CBM3a) . The lines 671-6, 671-7, 668-10 and 681-3 (Figure 3-3 CBM3a) displayed rays directed to the outside away from the seed, but with no other distinctive arrangement differences. Line 669-9 displayed a very distinct arrangement like a beehive (Figure 3-3, 669-9-CBM3a). Line 673-1 displayed a well labelled tadpole-like pattern with clear outlines (Figure 3-3, 673-1-CBM3a). Similarly, Phan *et al.*, (2016) observed differences in the labelling patterns and intensity using CBM3a among different *Plantago* species.

The staining patterns for CW and CBM3a suggests that there is less cellulose in the mucilage layer closest to the seed but more towards the outer areas of the seed mucilage. This means that there is a difference in the types and arrangement of polysaccharides in the mucilage layer close to the seed when compared to the outer layer of mucilage. Similarly Phan *et al.*, (2016) observed differences in glucan staining of *Plantago* species, with *P. debilis*, *P. coronopus* and *P. major* showing more intense

staining away from the seed coat. The differences in the staining intensity and patterns between mutants with CW and CBM3a suggest variations in amount and distribution of cellulose within the mucilage. For example, 671-7 (Figure 3-3, 6717-CBM3a and CW) with less intense overall staining may indicate lower levels of cellulosic polysaccharides compared to more intensely stained 673-1 (Figure 3-3, 6717-CBM3a and CW). The differences in the patterns of staining indicates differences in the manner in which cellulose is arranged in the mucilage.

CCRC M110, raised against *Phormium tenax* xylan, and found to bind strongly to *P. ovata* WT mucilage (Phan, PhD thesis 2018), was used for whole mount immunolabelling (Figure 3-3, CCRC M110). In WT a wide area was labelled with a "sea anemone" like pattern (Figure 3-3, WT-CCRC M110). In line 671-6 there was labelling around the seed, but no discrete patterns were observed, other than a fluorescent halo around the seed (Figure 3-3, 671-6-CCRC M110). For line 671-7 a "flame-like" pattern was observed leading out from the seed towards the edge of the mucilage (Figure 3-3, 671-7-CCRC M110). For lines 673-1, 668-10, 681-3 and 669-9 (Figure 3-3, CCRC M110) again the sea anemone-like labelling was observed. However, in lines 673-1 and 668-10 (Figure 3-3, 673-1, 668-10-CCRC M110) these structures seemed to be smaller, more compact and regularly arranged compared to the WT. The differences in the staining patterns of CCRC M110 indicates differences in the deposition of heteroxylan in the mucilage, but the nature of these remain unknown. All mutants and WT were stained with CCRC M110 which indicates the presence of heteroxylan epitopes in all of them. Except for lines 671-6 and 671-7 other mutants in the study along with WT showed a similar "sea anemone" like pattern (Figure 3-3, CCRC M1110) indicating the heteroxylan in the mucilage is arranged in a regular manner with lobular structures, where outer edges of the lobes are more intensely stained, possibly due to higher amount of heteroxylan or due to the lobular structure giving more opportunity for effective binding of the antibodies. The difference in the staining of 671-6 and 671-7 indicate that these sister lines have lost the characteristic arrangement of heteroxylan around the seed or even that the antibodies are unable to penetrate the mucilage structure which seems to be less organized in comparison to the WT. Similarly Tucker *et al.*, (2017) also observed differences in the labelling patterns of CCRC M110 for *P. ovata*

mutants including differences in the compactness. Phan *et al* (2016) and Cowley, O'Donovan and Burton, (2021) also observed sea anemone-like labelling in *P.ovata* with the LM11 antibody raised against wheat arabinoxylan and further noticed differences in xylan distribution among other *Plantago* species. The mutations in the lines studied here seem to have changed the spatial arrangement of the mucilage heteroxylan similar to the observations of Tucker *et al.*, (2017).

CCRC M129, raised against citrus pectin, was also used for whole mount immunolabelling (Figure 3-3, CCRC M129). The staining was not as intense as the labelling for xylan. A regular pattern was however observed in WT which appeared like "tadpoles"" swimming away from the seed (Figure 3-3, WT-CCRC M129). Similar to xylan labelling, pectin labelling was also highly disorganized with no patterns in line 671-6 (Figure 3-3, 671-6-CCRC M129). In lines 671-7 and 681-3 (Figure 3-3, 671-7, 681-3-CCRC M129) pectin was visualized as spokes radiating from the seed. In line 669-9 (Figure 3-3, 669-9-CCRC M129) there was an unstained area around the seed bordered by a narrow, stained area with a tadpole-like pattern. Mutants 673-1 and 668-10 (Figure 3-3, 673-1, 668-10-CCRC M129) also displayed a thick layer with a tadpole-like pattern, however the latter had an unstained ring around the seed. Although not quantitative, the lower intensity of the CCRC M129 labelling compared to CCRC M110 indicates presence of lesser quantities of pectin in the mucilage of *P. ovata* and its mutants used in this study. The labelling is lighter closer to the seed surface but darker in the outer layer of the mucilage indicating that the pectin present in the mucilage is concentrated in the outer layers of the mucilage. The weaker labelling such as for 669-9 (Figure 3-3, 669-9-CCRC 129) indicates a lower pectin content in the mucilage compared to the WT. Pectin is a viscosifying polysaccharide with gel forming properties (Gulfi, Arrigoni and Amadò, 2005) therefore the change in levels of pectin can give rise to differences in the rheological properties of the mucilage. Also pectin is a well fermented dietary fibre (Williams *et al.*, 2011) and changes in the amount as well as the deposition pattern in the seed mucilage may have an effect on their accessibility to microorganisms as well as fermentability during gastrointestinal fermentation.

These microscopy results indicate that the mutants show different arrangement of mucilage polysaccharides such as pectin, xylans and cellulose around the seed compared to the WT. The differences in the intensity of staining for each type of polysaccharide indicates differences in the amount of the particular polysaccharide within the mucilage, although these are not quantitative and so are only indicative. The differences observed in the staining between layers shows that there is variability in the composition of the L1, L2 and L3. The characteristic patterns of staining within layers of mucilage observed in mutants shows that not only has mutation changed the amount of polysaccharide and where it is present but it has also changed how it is organized within the structure of the mucilage. The normal deposition and arrangement of polysaccharides within the mucilage matrix has been shown to be just as critical at determining mucilage properties as composition in species like Arabidopsis (Sola, Dean and Haughn, 2019), although there is still much to be learnt about interactions between the polysaccharide components in the mucilage of most myxospermous species. However, although unknown in a physical sense, the differences in the composition, structure and arrangement of the various polysaccharides within the layers of mucilage may affect their properties and their applications in food and fibre, such as water affinity, interactions with other materials such as starch, viscosity, ease of extraction etc. This provides an opportunity to obtain novel properties in the mucilage compared to the WT which may be useful for creating variation in the applications of psyllium.

Mutants exhibit differences in mucilage yields and composition

Variation was observed in the yield of different mucilage fractions from the mutants (Figure 3-4A). Compared to the WT, lines 671-6 and 671-7 had significantly higher (P<0.05) total mucilage yield per gram of seed (Figure 3-4A). Lines 671-6 and 671-7 also yielded a significantly bigger CWE fraction compared to WT (P<0.05). The IAE fraction was significantly lower for 671-6 (P<0.05) compared to WT, making the mucilage yields of 671-6 and 671-7 stand out from that of all the other mutants and WT (Figure 3-4A).

When comparing the amounts of each mucilage fraction as part of the whole mucilage yield (Figure 3-4B), the ratios from mutants including 671-6, 671-7 and 673-1 were significantly different from WT ($P<0.05$) (Figure 3-4B). Lines 671-6 and 671-7 had significantly higher CWE% as a proportion of the total mucilage (TM). Line 673-1 had a significantly higher HWE% as part of the TM. However, the IAE% of the TM was the most different for lines 668-10, 671-6, 671-7 and 673-1, all being significantly lower than WT ($P<0.05$) (Figure 3-4B).

Cowley *et al.*, (2020) compared the mucilage fractions of six mutants to the WT and showed differences in the percentages of mucilage fractions from the WT. Similar to this study the IAE% was most reduced in the mutants in comparison to the WT suggesting a less robust mucilage envelope in these mutants that is less resistant to extraction.

Previous experiments clearly show that the six mutants analysed here show the presence of different polysaccharides such as pectin and xylan, differences in the mucilage structure and arrangement around the seed and variation in total mucilage yield as well as the amounts of mucilage fractions extracted under different temperatures. These data indicate there could be composition differences in the polysaccharides contained in the mucilage of these mutants. To further characterize the mucilage polysaccharides, monosaccharide analysis was carried out for PMP-derivatives of acid hydrolysed mucilage fractions for each mutant using RP-HPLC.

The mucilage from each mutant and the WT was extracted in three fractions CWE, HWE and IAE followed by determining the monosaccharide composition of each (Figure 3-5). It was observed that the monosaccharide composition of each fraction was different for each mutant and differences were observed between mutants as well as between mutants and WT.

Rhamnose, galacturonic acid, arabinose and xylose were the most common monosaccharides in all mucilage fractions of all mutants and WT (Figure 3-5, Supplementary Table 3-1). Fucose, glucose, ribose and mannose were not detected in the samples (Supplementary Table 3-1). A considerable amount of rhamnose and galacturonic acid were found in the CWE of all mutants and WT as shown in

(Figure 3-5 CWE), likely to be derived from pectin, as previously indicated using the ruthenium red staining as well as immunolabelling with the CCRC M129 antibody. All six mutants showed significant differences ($p < 0.05$) for all four monosaccharides when compared to the WT.

Lines 671-6 and 671-7 had a significantly lower rhamnose content compared to the WT. Galacturonic acid was significantly higher than WT in 673-1 while it was lower than WT in all other mutants. Both arabinose and xylose as well as X+A in CWE were significantly higher in line 671-6 while they were significantly lower in 668-10, 669-9 and 681-3 (Figure 3-5 CWE). The A/X ratios of CWE of all mutants was significantly different from that of WT ($P < 0.05$) (Figure 3-5 CWE, Supplementary Table 3-1) with line 671-6 having the highest A/X ratio (Figure 3-5, CWE-A/X) for CWE, indicating that it has more heavily substituted heteroxylan than the WT.

The rhamnose content of the HWE was significantly different in 668-10, 669-9 and 673-1 (Figure 3-5 HWE, Supplementary Table 3-1). Lines 668-10, 669-9, 671-6 and 681-3 all had a significantly lower galacturonic acid content than WT ($P < 0.05$). The X+A was significantly higher for line 668-10 compared to the WT ($P < 0.05$). The A/X ratio of the HWE was significantly lower in the lines 668-10, 669-9 and 673-compared to the WT (Figure 3-5 HWE) indicating that they are less substituted.

The IAE fraction of lines 669-9 and 673-1 contained significantly higher levels of rhamnose than the WT (Figure 3-5 IAE, Supplementary Table 3-1). Lines 671-6 had the highest galacturonic acid content in IAE out of all the lines. The X+A of all mutants except 681-3 was significantly different to that of WT ($P < 0.05$) (Figure 3-5 IAE) with line 671-6 having a much higher X+A than WT. The A/X ratios of all mutants were significantly different from that of WT ($P < 0.05$), with lines 669-9 and 673-1 lower and lines 668-10, 671-6, 671-6 and 681-3 higher, indicating that the substitution levels of IAE of all mutants are different to that of the WT (Figure 3-5 IAE, Supplementary Table 3-1).

 The results of the monosaccharide analysis of mucilage fractions from sequential mucilage extraction shows that monosaccharide profiles of mutants are different to that of WT, 671-6 being the most different. The A/X of the CWE and IAE of all mutants had more drastic changes compared to the A/X

ratio of the HWE (Supplementary Table 3-1) which suggests that mutations have affected the polysaccharides in the CWE and IAE to a larger degree, with varied substitution patterns compared to those of WT.

It should be noted that the total weight of the monosaccharides did not add up to the initial input (Supplementary Table 3-1). There were some unknown peaks observed during the RP-HPLC analysis which may be other components such as proteins and phenolic compounds. Also, it should be taken into consideration that some mass loss is routinely observed in monosaccharide quantification using this method. Phan *et al.* (2016) observed differences in the monosaccharide compositions of whole mucilage extractions from various *Plantago* species. Guo *et al.*, (2008), Ren *et al.*, (2020), Yu *et al.*, (2018) also observed differences in the monosaccharide profiles and the A/X ratios of *P.ovata*. Cowley, O'Donovan and Burton, (2021) highlighted that among the *Plantago* genus there are not only differences in the amount of CWE, HWE and IAE fractions but also some differences in the monosaccharide composition of mucilage fractions of each species studied.

Similarly, there is variation between the percentages and composition of each fraction of the mucilage of the *P.ovata* mutants studied here as shown in (Figure 3-5). The differences between the A/X ratios and other monosaccharide contents means that there may be differences in the length and composition of the main polysaccharide chains as well as changes in the degree of decoration of the main chain by side chains. It was found that all mutants had an increasing A/X correlating to IAE>HWE>CWE (Supplementary Table 3-1). Yu *et al.*, (2017) who fractionated the mucilage from *P. ovata* also observed an increase in the A/X ratios with increasing difficulty in extraction, similar to the mutants and WT in this study. This shows that the IAE fraction is more heavily substituted than the CWE fractions. It is noteworthy that the line 671-6 which had the highest A/X in IAE (Figure 3-5 IAE) also had the lowest IAE yield (Figure 3-5A) as well the IAE% of the TM. Therefore, it can be hypothesized that the IAE fraction of 671-6 is much more heavily substituted than the WT and adheres closely to the seed making it more resistant to extraction. However, more sophisticated structural analyses like linkage analysis or

NMR, plus potentially atomic force microscopy *in situ*, amongst others, would be needed to confirm these results. The changes in A/X ratio may also lead to differences in the viscosity, rheological properties and solubility in water at different temperatures (Guo *et al.*, 2008; Ren *et al.*, 2020; Yu *et al.*, 2018) which in turn would be a major factor that affects the applications and functionality of these materials in food systems.

Mutant mucilage shows different functional properties

Water absorption capacity (WAC) was determined by comparing the dry weight of seeds with the swollen weight (Cowley *et al.*, 2022). The WAC was significantly higher (P<0.05) in lines 669-9, 673-1 and 681-3 (Figure 3-6A). The total mucilage yields, including CWE, HWE and IAE of lines 669-9, 673-1 and 681-3, were not significantly different from the WT (Figure 3-4A) therefore the higher WAC is not due to higher mucilage content in the seed. Therefore, it can be hypothesized that the mucilage in these lines have a higher water affinity and are capable of binding more water per unit weight of mucilage. Higher WAC could make them potentially better candidates for the most common use of psyllium as a faecal bulker (McRorie *et al.*, 2021), with a smaller amount of mutant psyllium to get the same faecal bulking effects as the WT. It is also possible that the lines with higher WAC have a more robust mucilage network compared to the WT and this has been associated with thriving of seeds in adverse environmental conditions (Witztum, 1978; Gutterman & Shem-Tov, 1996). Therefore, it is possible that lines 669-9, 673-1 and 681-3 could have better germination success and ability to prevail under unfavourable conditions such as salinity, drought or higher temperatures.

Starch swelling power was determined by comparing the dry weight of starch in comparison to the swollen weight of starch granules in the presence of mucilage (Liu, Eskin and Cui, 2003). All mutants had a significantly lower starch swelling power (P<0.05) compared to WT-2019 grown under identical conditions (Figure 3-6B). The SSP of the control was not significantly different to the starch hydrocolloid mixes made with mucilage from lines 669-9, 671-6, 671-6 and 671-7. However, the presence of 673-1 and 681-3 mucilage led to a reduction in the SSP (Figure 3-6B). When the WAC and SSP were

correlated to each other a weak negative correlation was found between them (Pearson R= -0.495, sig=0.0213). The WT 2019 increased the SSP significantly to that of the control (P<0.05), while lines 673-1 and 681-3 led to a significant reduction of the SSP when compared to the control (P<0.05). Therefore, it can be hypothesized that the interactions between starch-mucilage, starch-starch, starch-water, mucilage-water could be different between the WT-2019 and the mutants 673-1 and 681-3. Ren, Linter and Foster, (2021) stated that in the presence of mucilage there may be a competition for water and volume between the starch and psyllium polysaccharides. The weak negative correlation between WAC and SSP could mean that the higher swelling capacity due to a high-water affinity of the mucilage of 673-1 and 681-3 could be reducing the available water for the starch granule swelling thereby causing competition for water among them, leading a reduction of the SSP. The differences in the WAC and SSP could be related to the differences in the composition of the mucilage. Changes in the monosaccharide profile, A/X ratios and percentages of different mucilage fractions would affect their solubility in water as well as their interaction with other molecules such as starch.

A study where the SSP of a blend of wheat and potato starch was determined in the presence of different concentrations of HPMC, gum arabic, pectin and konjac glucomannan, found no significant differences in the SSP of hydrocolloid-starch blends as compared to the control (Varela, Navarro and Yamul, 2016). In a different study using rice starch and hydrocolloid mixtures, guar gum, gum arabic and xanthan decreased the swelling power of rice starch at a 0.05% concentration while gellan gum increased the swelling power (Song *et al.,* 2006). It is suggested that at the lower concentration of hydrocolloids the increase in the viscosity in the continuous phase due to the presence of hydrocolloids inhibits the penetration of water into the starch granules leading to reduction in SSP. At higher concentrations of hydrocolloids or in highly viscous material like gellan gum the excessive viscosity leads to an increased sedimentation volume, thereby giving an increased SSP value (Song *et al.,* 2006). Therefore, it appears that the type of starch, viscosity, type of hydrocolloid as well as their concentrations have an impact on the SSP. Here, differences in the SSP of the mutants and WT (Figure 3-6B) indicate that they interact differently with starch. In order to further investigate the effects of

mutant mucilage on rice starch and rice flour pasting behaviour, rapid visco analysis (RVA) was carried out.

Mutant mucilage leads to changes in pasting properties of rice flour and rice starch

Rapid visco analysis was carried out to investigate if the addition of mutant mucilage could change the pasting properties of rice flour and rice starch as compared to WT (Figure 3-7, 3-8, Table 3-2, 3-3). Husk fractions at a level of 1% were mixed with rice flour or rice starch for this analysis.

The addition of *P.ovata* mucilage from all mutants and WT led to a slight but significant ($p < 0.05$) reduction of pasting temperature of rice flour-mucilage blends compared to the rice flour control without any hydrocolloid addition (Table 3-2) ranging from 1.4 °C to 2.45 °C. However, there were no significant differences in the pasting temperatures among the WT and mutants.

Pasting temperature is the temperature at which highest viscosity is observed in the gelatinization curve. During this process the starch granules absorb water and swell, finally rupturing to release amylose and viscosifying the medium (Sandhu, Singh and Malhi, 2007). At the gelatinization point the leached amylose and low molecular weight amylopectin form a continuous phase which is dispersed with swollen starch granules. During gelatinization in the presence of polysaccharides like psyllium mucilage a phase separation occurs where there is a continuous phase rich in hydrocolloids and a dispersed phase rich in amylose and amylopectin (Alloncle and Doublier, 1991). In the presence of hydrocolloids the gelatinization of starch occurs in **wa**ter deficient conditions because the water holding ability of hydrocolloids can reduce the effective water concentration especially when the concentration of starch and hydrocolloid is high and/or the water is lower (Sikora, Kowalski and Tomasik, 2008). The leached amylose and low molecular weight amylopectin interact with the hydrocolloids and depending on the types and amount of hydrocolloids added, the pasting temperature and other pasting properties can be altered (Shi and BeMiller, 2002). During the initial stages of starch swelling small amounts of amylose could be leached into the medium which will interact with the hydrocolloid, hastening the pasting (Díaz-Calderón et al., 2018). Similar interactions of the polysaccharides in the *P. ovata* mutant and WT

mucilage with the starch granules in the rice flour hydrocolloid blend has led to a reduction in the pasting temperature.

Peak viscosity is the highest viscosity reached during gelatinization. Peak viscosity was increased in mucilage-rice flour blends in comparison to the control sample (Figure 3-7, Table 3-2). The peak viscosity of line 673-1 was significantly lower than the WT (P<0.05) however all other mutants showed a similar peak viscosity to that of WT. Similar to these findings, Mancebo *et al.*, (2015) and Cappa, Lucisano and Mariotti, (2013) also found an increase in peak viscosity with the addition of psyllium into rice flour pastes, caused by the high water binding ability of strongly hydrophilic psyllium polysaccharides. The increased viscosity is caused due to interactions such as starch-starch, hydrocolloid-hydrocolloid (homotypic) and starch-hydrocolloid (heterotypic) (Alloncle and Doublier, 1991; Sullo and Foster, 2010; Christianson *et al.*, 1981). All mutant and *P. ovata* WT mucilage used in this study were viscous in nature with water binding ability and similar interactions with starch granules and mucilage polysaccharides therefore led to a significantly higher peak viscosity. That is, the change in peak viscosities of mutant and WT mixtures are likely to be due to similar homotypic and heterotypic interactions even though there are differences in their monosaccharide composition and A/X ratios. The way the complexity of heteroxylan influences how it behaves in such starch based mixtures is an area for further study.

Once the highest viscosity is reached, the viscosity then drops due the effect of shear stress and this is called the trough viscosity. The trough viscosity of all rice flour-mucilage combinations was higher compared to the control however the mutants and the WT has no significant differences between them (Table 3-2). Breakdown viscosity is the difference between the peak viscosity and trough viscosity. The breakdown viscosity of all mucilage-rice flour blends was higher than the control (Table 3-2, Figure 3-7). However, the breakdown viscosity was also similar in all mutants and WT except for line 671-6, which had a significantly lower breakdown viscosity. The water absorbed by starch granules during

gelatinization leads to melting of the crystalline region, causing rapid entry of water into the granule causing rapid swelling before the granules eventually rupture and disintegrate. This reduction in occupied volume after the granules disintegrate thereby leads to a drop in viscosity during the holding phase. The lowest point reached during this phase is the trough viscosity (Batey, 2007; Alloncle, 1989). Trough and breakdown viscosities are affected by various factors; starch paste composition, force used for shearing, type of starch and temperature (Schirmer *et al.*, 2013; Copeland *et al.*, 2009). The reduction of water available for granule swelling due to the high-water affinity of hydrocolloids increases the shear force exerted in the starch granules during this phase, thereby increasing the breakdown in the presence of hydrocolloids (Satrapai and Suphantharika, 2007). The mucilage polysaccharides of the *P. ovata* WT and mutants used in the study have high water holding capacity which reduces the water available for the starch granules in the rice flour paste thereby increasing the shear forces leading to higher trough and breakdown viscosities.

Final viscosity is the viscosity at the end of the cooling period of RVA. All mucilage additions increased the final viscosity in mucilage-rice flour pastes compared to the control (Table 3-2, Figure 3-7) however again there were no significant differences between the WT and mutants (P>0.05). Setback viscosity is the difference between the final viscosity and trough viscosity (Zhou *et al.*, 2007). All mucilage additions increased the setback viscosity compared to the control (Table 3-2, Figure 3-7). However, there was no significant difference (P>0.05) of the setback between the mutants and WT (Table 3-2, Figure 3-7). The end holding period at high temperature is followed by a cooling period at which stage retrogradation occurs by realigning of amylose and amylopectin to form crystalline structures (Batey, 2007; Kaur *et al.*, 2007). During the cooling of the rice starch paste it forms a gel with polymer complexes with the amylose and low molecular weight amylopectin extruded from the starch granules during gelatinization and this gelation is modified by the addition of hydrocolloids, thereby increasing the viscosity of systems with added hydrocolloids (Collar, 2003). Similarly, addition of mucilage led to an increase of both final and setback viscosity. Increases of final and setback viscosity were also observed with addition of

psyllium husk to rice starch by Cappa, Lucisano and Mariotti, (2013). The mucilage further strengthens the network formation of amylose and amylopectin thereby forming a stronger gel with higher viscosity.

Similar to the observations for mucilage-rice flour pastes the mucilage-rice starch also had lower pasting temperatures compared to the control sample (Figure 3-8, Table 3-3). The peak viscosity and trough viscosity were significantly higher in the rice starch pastes with added mucilage, with line 671-6 showing the highest viscosity. The breakdown viscosity was significantly higher for line 671-7 when compared with WT (Table 3-3). The final viscosities of mutants and WT were not significantly different from each other (P>0.05). The setback viscosity was significantly higher for line 671-7 compared to the WT (Table 3-3).

All mucilage additions modified the pasting curves of rice flour and rice starch (Figure 3-7, 3-8, Table 3-2, 3-3) compared to their respective control samples. Although there were differences in the pasting temperatures and viscosities, the shape of the pasting curve only showed subtle differences between that for WT or for the mutants, for both rice flour and rice starch (Figure 3-7, 3-8).

In all curves of rice flour paste with added mucilage a second peak was observed after the peak viscosity (Figure 3-7). This is known as the "atypical peak" and will be discussed in more detail in Chapter 4. Mutant lines 671-7 and 668-10 had rice flour pasting profiles very similar to WT. For 681-3, 673-1 and 669-9 the atypical peak was observed in rice flour pastes (Figure 3-7) but it was of a lower intensity compared to WT and other mutants. For line 671-6, the gelatinization curve was shifted slightly to the right, that is gelatinization was delayed slightly compared to WT (Figure 3-7). Also, the atypical peak was weaker than WT although still detectable. In WT the highest viscosity or the final viscosity is reached gradually towards the end of the 2.5 min RVA run, however for 671-6 this occurred earlier, with a steep increase in viscosity followed by a plateau giving a different RVA profile from WT as shown in Figure 3-7. This plateau was also observed in the pasting of rice starch in the presence of 671-6. Mutant Line 671-6 displays the highest deviation of pasting profiles for both rice starch and rice starch pasting from that of WT (Figure 3-7, 3-8) and is therefore the most prominently different from the WT.

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The pasting parameters of rice flour pastes were correlated to the mucilage monosaccharide composition (Table 3-4). The pasting temperature was positively correlated with galacturonic acid content of the CWE while it was negatively correlated with the rhamnose content of the IAE fraction.

Similar correlations were also carried out for rice starch pastes (Table 3-5). A higher number of significant correlations were observed between the pasting properties of rice starch and the mucilage monosaccharides, possibly due to the purer nature of the rice starch in comparison to the rice flour (Table 3-5). The breakdown viscosity as well the setback viscosity of rice starch was negatively correlated with the A/X ratio. The increase in the branching of the heteroxylan has reduced the gelling of the starch paste. The gel network is formed by the interaction of hydrocolloids with the amylose and amylopectin, however with the increasing A/X of the CWE it is possible that the heteroxylan from the mucilage self-associates rather than strengthening the network of amylose and amylopectin. The results of correlations show that the monosaccharide composition of the mucilage affected the pasting properties although overall the modification of the pasting curves is subtle when compared with the WT. The trough viscosity of rice starch was positively correlated to the arabinose, xylose and X+A of the IAE fraction. Higher X+A indicates a higher arabinoxylan content in the IAE fraction. Line 671-6, which had the highest X+A in the IAE fraction, also produced the highest trough viscosity in rice starch pastes. This could mean that higher heteroxylan content in the IAE fraction reduces the shear thinning thereby maintaining a higher trough viscosity.

The monosaccharide content of mutant line 671-6 differed from other mutants, having a higher X+A as well as A/X ratios in the CWE and IAE fractions. The different RVA profile for both rice starch and rice flour during the last stage of the gelatinization compared to the WT and the other mutants could be due to this difference in the polysaccharide composition and structure in CWE and IAE, which alters how it forms homotypic and heterotypic interactions. Although the other mutant lines also displayed differences in the monosaccharide profiles to that of the WT it appears that these differences are not strong enough to create large modifications to the pasting profiles in both rice flour and rice starch.

Emulsification and foaming properties

The emulsion capacity (EC) of mucilage was studied as shown in Figure 3-9A, B. All mucilage increased the EC at 30 min compared to the control. Lines 669-9, 673-1 and 681-3 had significantly lower ($P<0.05$) EC at 30 mins, although the differences were subtle (Figure 3-9A). At 60 mins EC was similar in all mutants and WT except for line 671-6 (Figure 3-9B) which had a lower EC. The emulsion stability (ES) at 30 mins was significantly higher than the control but not significantly different from the WT (Figure 3-9C) while the control separated completely into oil and water with an ES of zero. This shows that the mucilage from *P. ovata* WT and mutants studied here are capable of maintaining emulsions, although none of the mutants performed better in comparison to the WT (Figure 3-9, A, B, C). Amiri Aghdaei *et al.*, (2012) used psyllium as a fat mimetic in low fat mayonnaise with sensory acceptability correlating to flavour and texture. The findings of emulsification properties in the our study also suggest that psyllium can be used for maintaining emulsions with stability under heat treatments - a property which can be exploited in the food industry.

The foaming capacity (FC) was significantly higher than WT for line 671-6 (Figure 3-9D). Lines 668-10 and 669-9 had zero FC. Although some foaming was seen for the other four mutant extracts and the WT, it disappeared quickly and all had a foam stability of zero. Therefore, neither the WT nor the mutants appear to be suitable for applications of foaming.

The functional properties WAC, SSP, EC 30, EC 60, ES 30 and FC were correlated to the monosaccharide composition of mutants (Table 3-6). No significant correlations were found between the monosaccharide composition and the WAC, SSP and EC 30. The EC 60 was negatively corelated to the arabinose, xylose and X+A of the IAE fraction. The ES30 was negatively correlated to the arabinose and A/X of the CWE and arabinose and X+A of the IAE, while positively correlated to rhamnose. The FC was positively corelated with the xylose, arabinose and X+A of the CWE and the IAE (Table 3-6). A negative correlation was found between ES30 and FC (R= -0.821, Sig= 0.024). Given that arabinose and xylose are the main components of heteroxylan, these correlations show that the higher

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heteroxylan in mucilage is associated with the formation of unstable foams while reducing the ability of maintaining stable emulsions. Line 671-6, which had a significantly higher X+A in the CWE and HWE (Supplementary Table 3-1) with the highest A/X ratio in CWE (Figure 3-5, CWE), was the only mutant to show a lower emulsion capacity at 60 mins in comparison to the WT (Figure 3-9B) but there was a large standard deviation in the ES at 30 minutes (Figure 3-9C). Therefore, it can be hypothesized that increasing amounts of heteroxylan in CWE and IAE and the higher degree of substituted (A/X) polysaccharides in the CWE in the psyllium reduces its ability to maintain stable emulsions. It is possible that highly branched heteroxylan in the CWE form self-associations leading to the reduction of stability of the emulsion.

In vitro fermentation of *P. ovata* mutants

Wild type *P.ovata* (M1) and four mutants 671-6 (M2), 671-7 (M3), 673-1 (M4) and 668-10 (M5) were chosen for *in vitro* fermentation trials using a human faecal inoculum. The fermentation was set up and run with the aim to provide data on gas, SCFA and NH_3 amounts and changes in the pH were measured. However, although gas data recording was carried out for this study, the gas kinetics will not be reported or discussed in this section due to data loss caused by a state-wide power cut, as described in Chapter 1. The end products of fermentation of the substrates will be reported in this section.

Table 3-7 and Figures 3-10 and 3-11 show end products of the *in vitro* fermentation. When comparing the end products of M(n)-W and M(n)-F together for the five different lines, all lines produced similar amounts of acetate ($P>0.05$). M2 yielded the highest propionate but it was not significantly different from M1 and M3 (Table 3-7). M1 produced the highest butyrate, however it was not significantly different from M2, M3 or M4 ($P>0.05$). The highest amount of branched chain SCFA, as well as highest branched proportion, was produced by M3, which was significantly higher than M1, M2, M4 and M5 ($P<0.05$). The highest total SCFA was produced by M2, however, it was similar to M1, M3 and M4. All lines produced similar amounts of NH_3 ($P>0.05$) (Table 3-7 and Figure 3-11). M2, which yielded the highest total SCFA, also produced a significantly lower pH than the other substrates ($P<0.05$) (Table 3-7). When comparing

the M(n)-W and M(n)-F it was found that M(n)-W yielded higher acetate, propionate, butyrate, and total SCFA amounts as well as NH$_3$. The branched chain SCFA and pH were similar for all M(n)-W and M(n)-F (Table 3-7).

The total SCFA production for M1-W, M4-W and M5-W continued to increase until 72 h. Total SCFA production plateaued for M2-W at 48 h and M3-W at 24 h (Figure 3-10). Similar trends were observed for acetate and propionate also.

Samples M1-F, M3-F, M4-F and M5-F continued to produce total SCFA until 72 h although M2-F plateaued at 48 h. For M2-F, M3-F and M5-F (Figure 3-10) the total SCFA production slowed down between 12 h – 24 h, followed by an increase from 24 h onwards. For all substrates Acetate>Propionate>Butyrate>Branched SCFA. There was higher production of NH$_3$ by the M(n)-W than the M(n)-F during the course of fermentation which is observed very clearly in M1 (Figure 3-10) however at the end point, both M(n)-W and the M(n)-F were similar in M2, M3, M4 and M5. For all M(n)-W the NH$_3$ increased until 24 h and then reduced past this point. M2-F, M4-F and M5-F showed a similar trend of NH$_3$ production, with an increase from 0h to 4 h, a decrease from 4 h to 12 h, an increase from 12 h to 24 h, a decrease from 24 h and another increase from 48 to 72 h (Figure 3-`10).

The higher production of total SCFA in M(n)–W compared to M (n)-F means there was access to more fermentable substrates to facilitate the production of SCFA. The removal of the CWE fraction from the M(n)-F prior to the experiment led to a reduction in fermentable carbohydrates. As shown in Supplementary Table 3-1 the A/X ratio of the CWE fraction of all the lines was lower in comparison to their HWE and IAE fractions, which means the polysaccharides are less complex in their branching. It can be hypothesized that the less complex branched polysaccharides in the CWE are more accessible to the bacteria for fermentation, leading to a higher production of SCFA. Th greater fermentability of the CWE could also be due to the differences in the sugar composition and higher amounts of pectic polysaccharides in the CWE. Marlett and Fischer, (2002) who fermented fractionated psyllium gel into A (alkali insoluble), B (gel forming consists of 55%) and C (viscous soluble fraction <15%) found A

unfermentable, B poorly fermentable and C rapidly and almost completely fermentable by 72 h. The viscous soluble fraction is similar to the CWE in this study and the removal of this more fermentable fraction led to the reduction of total SCFA similar to the study reported by Marlett and Fischer, (2002).

The plateauing of M2-W and M3-W while M1-W, M4-W and M5-W continued to produce SCFA suggests that there are differences in the polysaccharide composition in each of the five lines. There are some polysaccharides which very slowly ferment producing SCFA until 72 h and some others which ferment and exhaust by 24 h or by 48 h. The lines used in this study display differences in their monosaccharide composition and A/X ratios (Supplementary Table 3-1) which are hypothesized to translate as differences in length of polysaccharides, type and number of branches etc. as discussed earlier. The differences in fermentability of the five lines, even though they are quite subtle, strengthens the idea that the polysaccharides in the mucilage of the WT *P. ovata* and mutant *P. ovata* are structurally different, and thus differentially accessible to the microbial population.

NH_3 is a toxic metabolite which is produced as a result of fermentation of protein, including that contained in dead bacteria, which is detrimental to health (Macfarlane and Macfarlane, 2012). The increase of NH_3 until 24 h in M(n)-W suggests a lack of fermentable carbohydrates, leading the bacteria to metabolize other sources. The decrease in NH_3 that follows could be due to adaptation of the microorganisms to the substrate which then leads to availability of carbohydrates for metabolization which reduces the metabolization of proteins. The increasing and decreasing trend in the NH_3 production of the M(n)-F suggests that there is a lack of fermentable carbohydrates in the periods where NH_3 increases, followed by an adaptation which reduces the NH_3 and the cycle then repeats itself. This is also an indication of the presence of different types of polysaccharides with structural variation, not all of which the microbes can access immediately to ferment, amongst *P. ovata* and the mutant lines studied here.

The fermentation end products were correlated with the monosaccharide composition, A+X and A/X of the of CWE, HWE and IAE of the lines used for the study (Table 3-8). A strong positive correlation

(R=0.957, sig=0.011) (Table 3-8) was observed between the arabinose content of the CWE and butyrate production. However, this does not agree with (Hu *et al.*, 2013) who states that fermentation of aldehydes mainly produce butyrate while fermentation of ketose sugars mainly produces acetate and propionate. A strong positive correlation was observed between the A/X ratio of the HWE and NH_3 (R=0.904, sig=0.035). The suggests that as the A/X ratio increases, the degree of branching increases, making the polysaccharides more difficult to ferment. Fractions with a high A/X ratio therefore contain less fermentable carbohydrates available in the medium leading to increases in protein fermentation and producing more NH_3. A strong positive correlation was observed between galacturonic acid in IAE and propionate levels (R=0.912, sig=0.031) (Table 3-8) suggesting the utilization of galacturonic acid as a substrate by the bacteria to produce propionate.

Plantago ovata, which is the source of commercially used psyllium, is quite poorly fermentable both *in vitro and in vivo* and mainly suitable for faecal bulking (Marteau *et al.*, 1994; Gunn *et al.*, 2020). Edwards *et al.*, (1992) found that about 50% of psyllium consumed by rats is excreted after partial fermentation. Under *in vitro* conditions Bifidobacteria were only able to ferment psyllium after simulated digestion (Elli *et al.*, 2008). The mutants (M2, M3, M4 and M5) fermented here showed very subtle differences in their SCFA production when compared with the WT *P. ovata* (M1) so it appears that these mutants are also poorly fermentable, although there are differences in the monosaccharide composition (Supplementary Table 3-1) and arrangement of different polysaccharides (Figure (3-3) in the mutants.

Conclusions and future directions

In this study mucilage from *P. ovata* mutants produced by gamma irradiation has been studied and compared to the wild type with the aim of identifying material which displays different properties and that may be exploited for applications in food and as fibre. Growing the mutant lines in the glasshouse showed that there are pleiotropic effects not just on mucilage characteristics but also on plant growth, seed viability and yield. This would have a direct impact on economic viability and would have to be considered if mutant *P. ovata* is to be grown for commercial purposes. Genetic studies are not included in the scope of this study, however desirable mutations could be identified by sequencing that would allow the development of markers for breeding, and in this way background mutations, not associated with the superior property, could be removed by backcrossing and phenotypic selection.

Microscopy methods using stains and immunolabelling with polysaccharide-specific antibodies were used to study mucilage around the seeds, which showed that the mutants exhibit differences in the arrangement and interactions of various polysaccharides such as pectin, cellulose and xylan in the mucilage and possibly have different amounts of them present too. This was further investigated by mucilage extraction using a fractionation pipeline which gave rise to varying mucilage yield as well percentages of fractions soluble at different temperatures. Monosaccharide analysis confirms that there are differences in the A/X ratios and monosaccharide composition of these various fractions. However, the main component of all mucilage is heteroxylan and it is this component which dictates behaviour in the various assays and in the fermentation experiments. There are differences in the main chains as well as the branching patterns in the mucilage from different mutants but microscopy and monosaccharide analysis provide only an indication of this. For more detailed analyses, and a clearer definition of the actual physical differences methods such as linkage analysis (Pettolino *et al.*, 2012) or Polysaccharide Analysis using Carbohydrate gel Electrophoresis (PACE) (Mortimer, 2017) is suggested.

Variations in the mucilage types are highlighted by their different water absorption capacity and effects on starch swelling capacity. Neither mutants nor WT formed stable foams but they were capable of

forming stable emulsions, although there were only subtle differences between them. Investigation of pasting properties showed that both mutants and WT alter the pasting profiles of rice flour and rice starch. However, the differences of the pasting profiles are not very distinct between WT and the mutants. This may suggest that although there is some structural alteration in the mutants due to mutations the effect is not strong enough to impart large differences in starch gel systems in comparison the wild type of psyllium. This in turn would not make them good candidates for application for further studies in starch-based food systems such as gluten free breads. However, the changes in the mucilage may be utilized for other purposes such as in fibre supplements, especially due to the higher WAC.
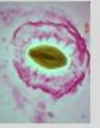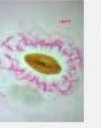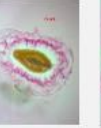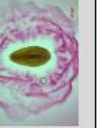
Mutant *P. ovata* display subtle differences in fermentability to the wild type but overall remain poorly fermentable. Mutant *P. ovata* maybe used for faecal bulking related uses similar to *P. ovata* wild type, and it appears that mutation may not be a suitable method for improving the fermentability. However, experiments using a larger number of mutant lines would have to be conducted to confirm this and the hypothesis that a visual phenotype translates to differences in physical properties may not be an accurate selection method. It is possible, but unlikely, that key chemical changes needed to improve foaming, emulsification and fermentability would not be visibly obvious. However, it is clear that the highly substituted nature of the heteroxylan remains the main driver of behaviour in aqueous systems and as such, major rather than more minor subtle changes are likely to be required to cause significant downstream alterations in psyllium properties.
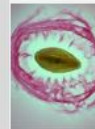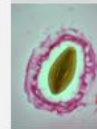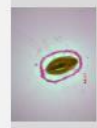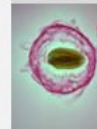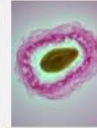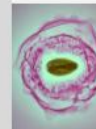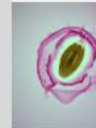
Acknowledgements

Figures

Table 3-1. Screening results for 25 *P. ovata* mutants and WT.

Mutant number	A) RR screening results	B) RR image	C) Water absorption capacity (% w/w)	Mutant number	A) RR screening results	B) RR image	C) Water absorption capacity (% w/w)
WT commercial	8/12 mutant		36.67±0.58	671-10	12/12 mutant		21.33±0.58
642-6	12/12 mutant		46.67±0.58	671-11	12/12 mutant		27.33±0.58
665-10	12/12 mutant		37.67±0.58	673-1*	11/12 mutant		34.67±0.58
668-10*	12/12 mutant		54.33±0.58	681-3*	12/12 mutant		52.67±0.58
669-9*	12/12 mutant		56.33±0.58	681-4	12/12 mutant		52.67±0.58
671-6*	12/12 mutant		24.67±0.58	836-2	4 /12 mutant		42.67±0.58
671-7*	12/11 mutant		38.67±0.58	836-10*	8/12 mutant		64.00±0

Mutant number	A) RR screening results	B) RR image	C) Water absorption capacity (% w/w)
838-10	9/12 mutant		64.67±0.58
839-2	7/12 mutant		60.00±0
840-9	5/12 mutant		52.67±0.58
841-8*	10/12 mutant		30.00±0
841-10*	12/12 mutant		25.33±0.58
842-11*	11/12 mutant		55.00±1.00

Mutant number	A) RR screening results	B) RR image	C) Water absorption capacity (% w/w)
858-4	7/12 mutant		53.67±0.58
870-3*	12/12 mutant		33.33±1.15
881-2	6/12 mutant		25.00±1.00
881-7	12/12 mutant		NA
871-8	9/12 mutant		52.67±1.15
882-9*	12/12 mutant		NA

A) Shows the number of mutants (n) observed across 12 biological replicates. The remaining number (12-n)/12 is the number with a WT phenotype observed using the naked eye. B) Representative mucilage phenotypes observed for 25 selected *P. ovata* M3 mutant lines in ruthenium stain using a dissecting microscope to observe the staining pattern, mucilage distribution and size of the mucilage envelope. Images are under 1.25X magnification. Scale is 0.5mm. C) Water absorption capacity (WAC). Values are mean and standard deviation of at least three replicates. The 12 mutant lines selected from the secondary screening are indicated by *.
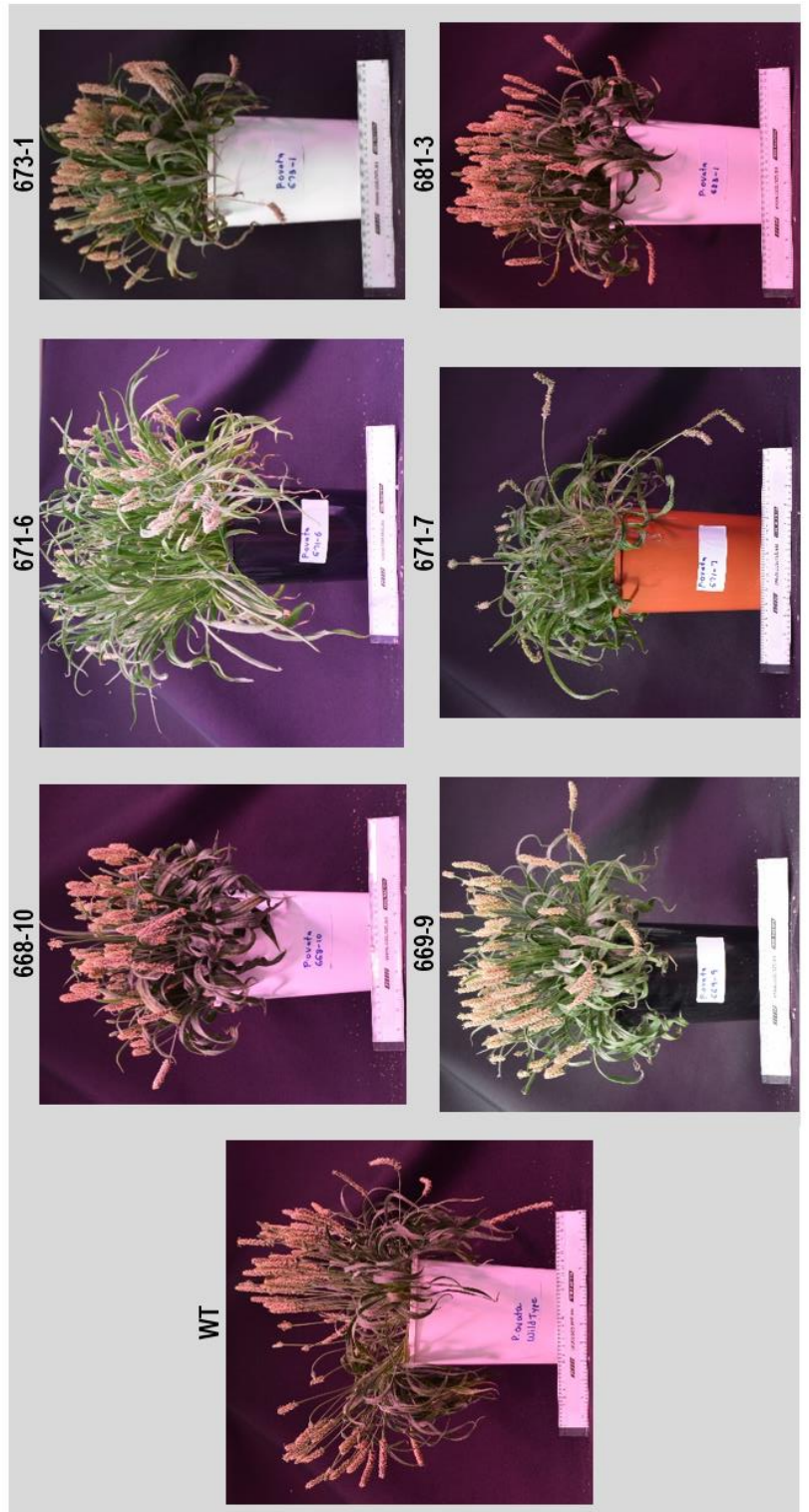
Figure 3-1. Plant phenotypes observed for 12 *P. ovata* mutant lines in comparison to the wild type *P. ovata* grown under identical growth conditions.
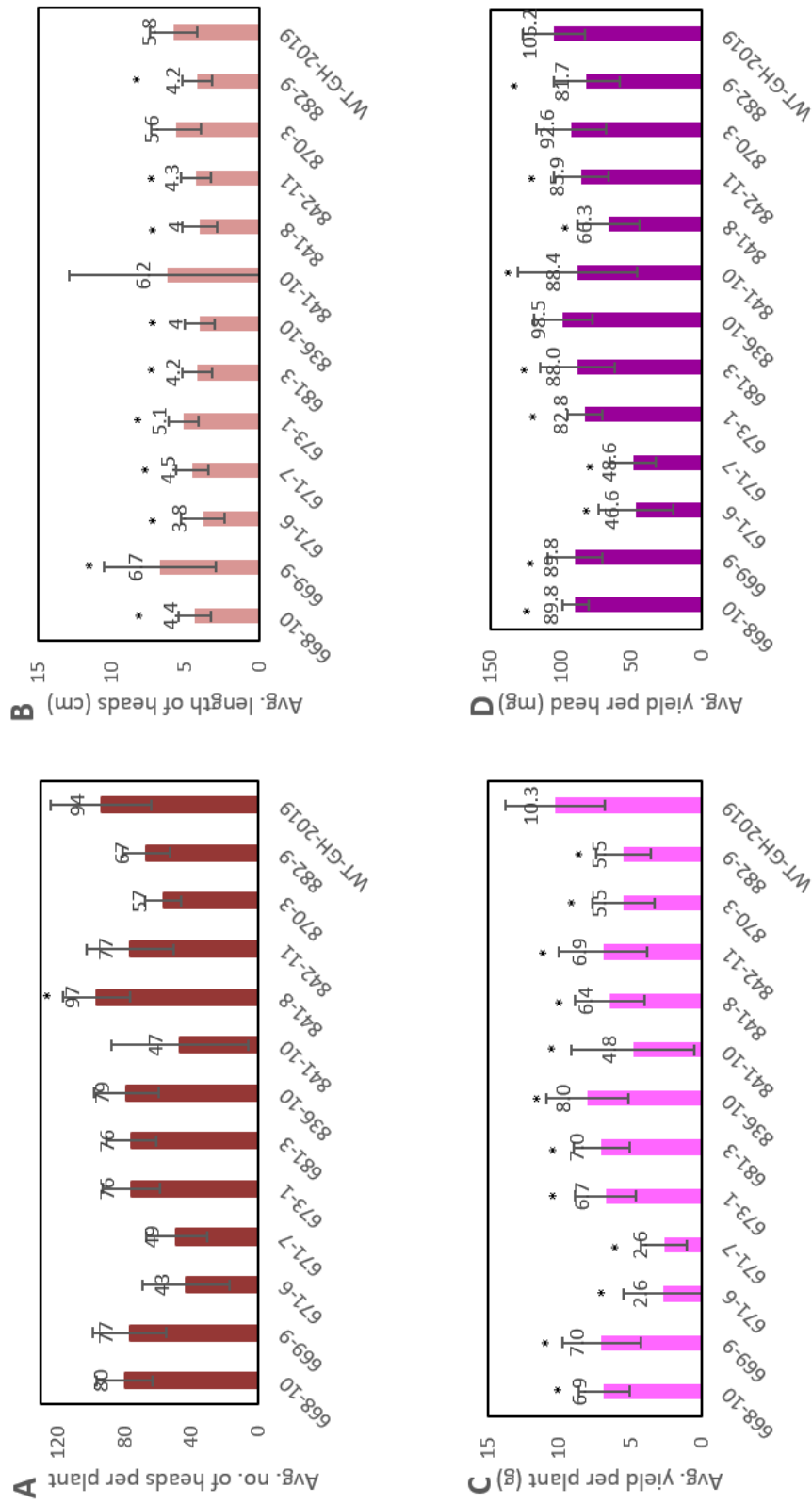
Figure 3-2. Agronomic traits of 12 *P. ovata* mutant lines. **A.** Seed yield (g) per plant. Values are mean and standard deviation of at least 12 biological replicates. **B.** Seed yield on individual heads of each plant. Values are mean and standard deviation of at least ten heads for at least 12 plants per mutant line. **C.** Heads per mutant plant. Values are mean and standard deviation of at least 12 biological replicates. **D.** Length of seed heads (cm). Length of seed head is the total distance of inflorescence with seed excluding the stalk. Values are mean and standard deviation of at least ten heads for at least 12 plants per mutant line. Means values which are significantly different to the WT at 95% confidence are indicated with * (p<0.05).
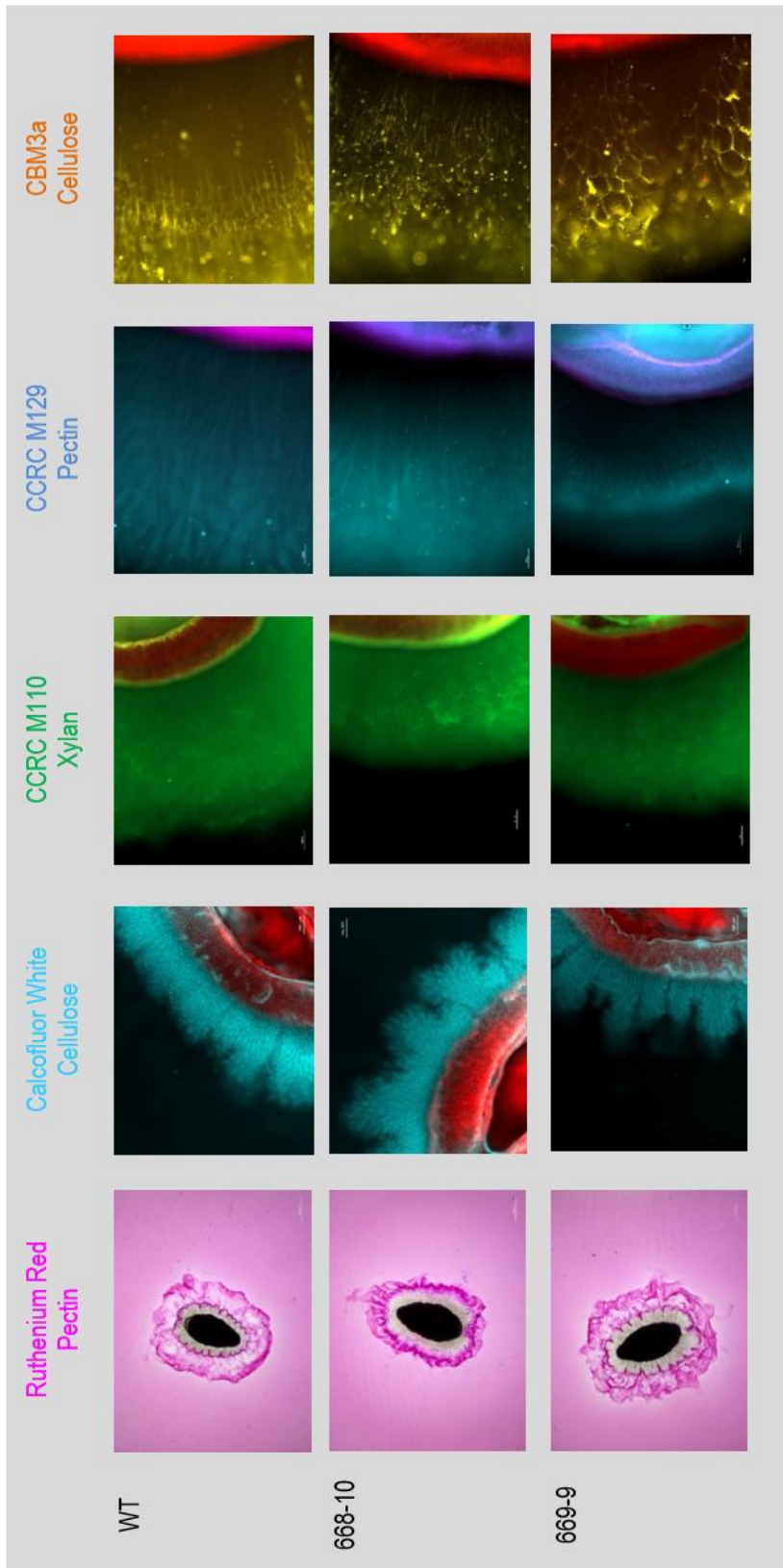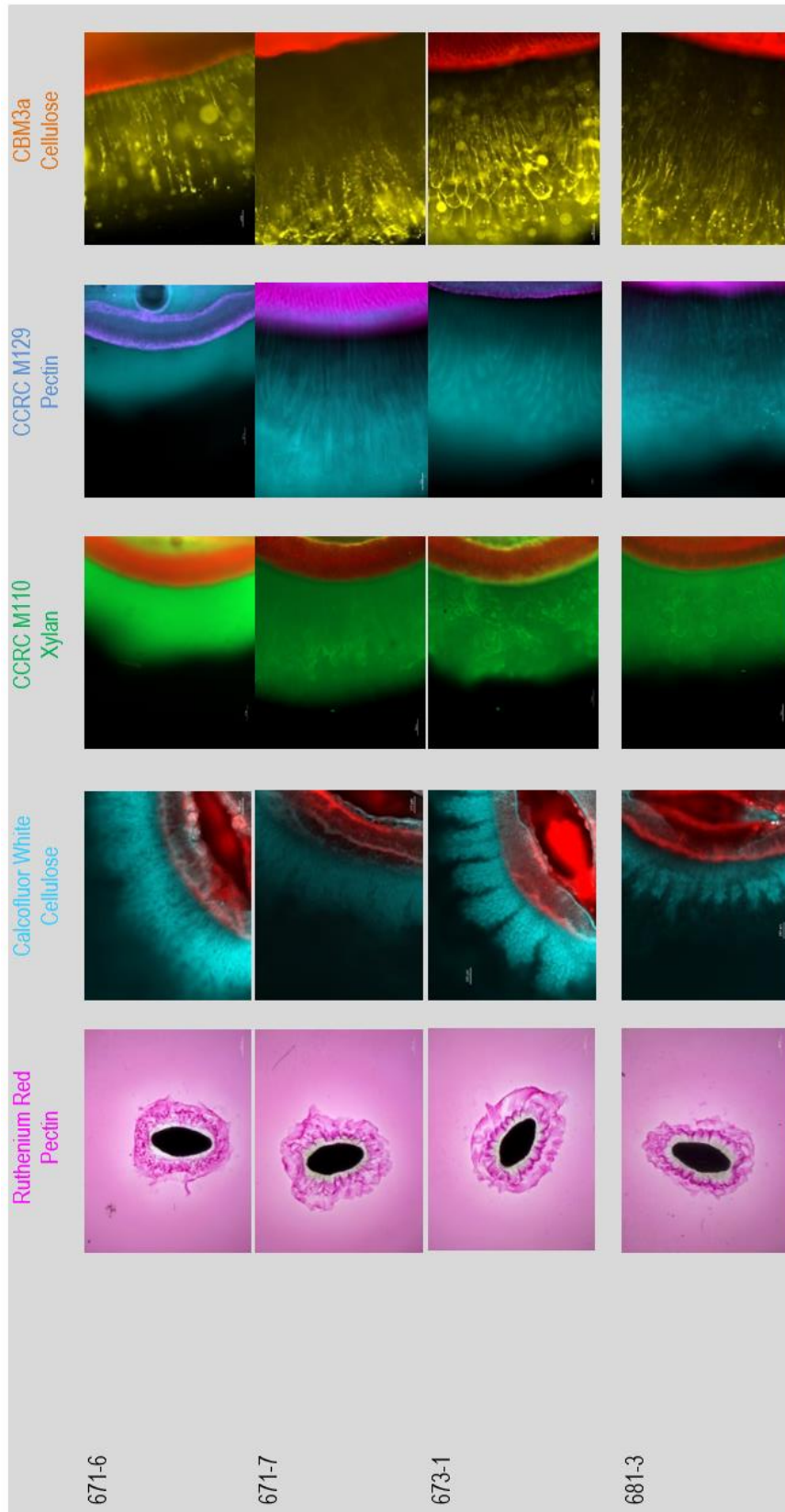
Figure 3-3. Expanded mucilage structure of six *P. ovata* mutants and wild type. Ruthenium red (dark pink) stains acidic polysaccharides at the mucilage periphery. Scale = 1 mm. Calcofluor white stains cellulose in the outer layers of mucilage. Scale =200 μm. Antibodies CCRC M110, CCRC M129, and the carbohydrate binding module CBM3a have been used for immunolabelling xylan, pectin and cellulose in the inner mucilage layers. The seed is counterstained with propidium iodide (red/yellow). Scale =200 μm
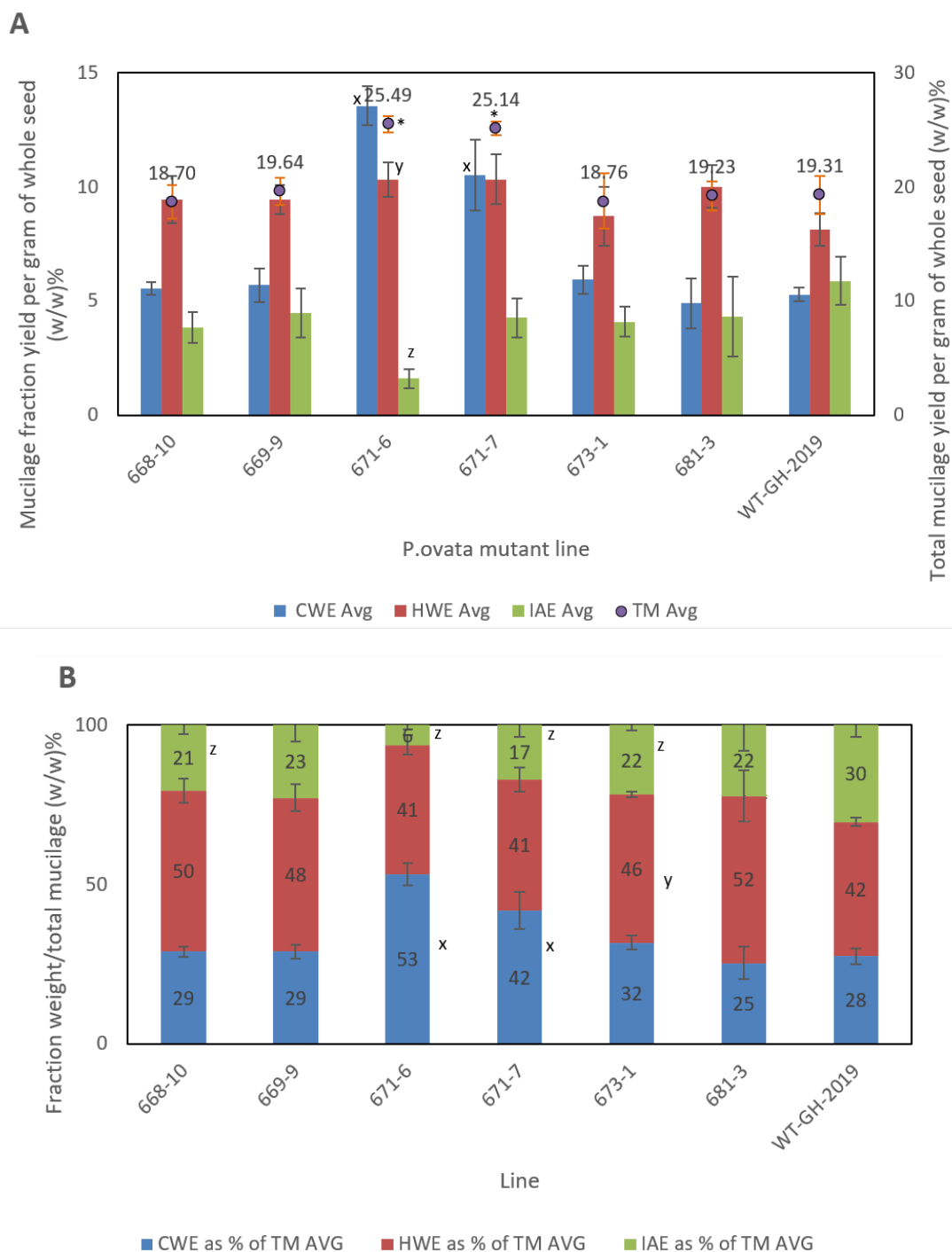
Figure 3-4. Mucilage yields of six *P. ovata* mutant lines. **A.** yield of each mucilage fraction and total mucilage (TM) yield as a share of whole seed weight. Values significantly different from WT are indicated by letters (P<0.05) **B.** Percentages of each mucilage fraction from total mucilage extracted. Values are rounded off to nearest integer. CWE = cold water extractable mucilage fraction (blue); HWE = hot water extractable mucilage fraction (red); IAE = cold water extractable mucilage fraction (green).Values significantly different from WT are indicated by letters (P<0.05).
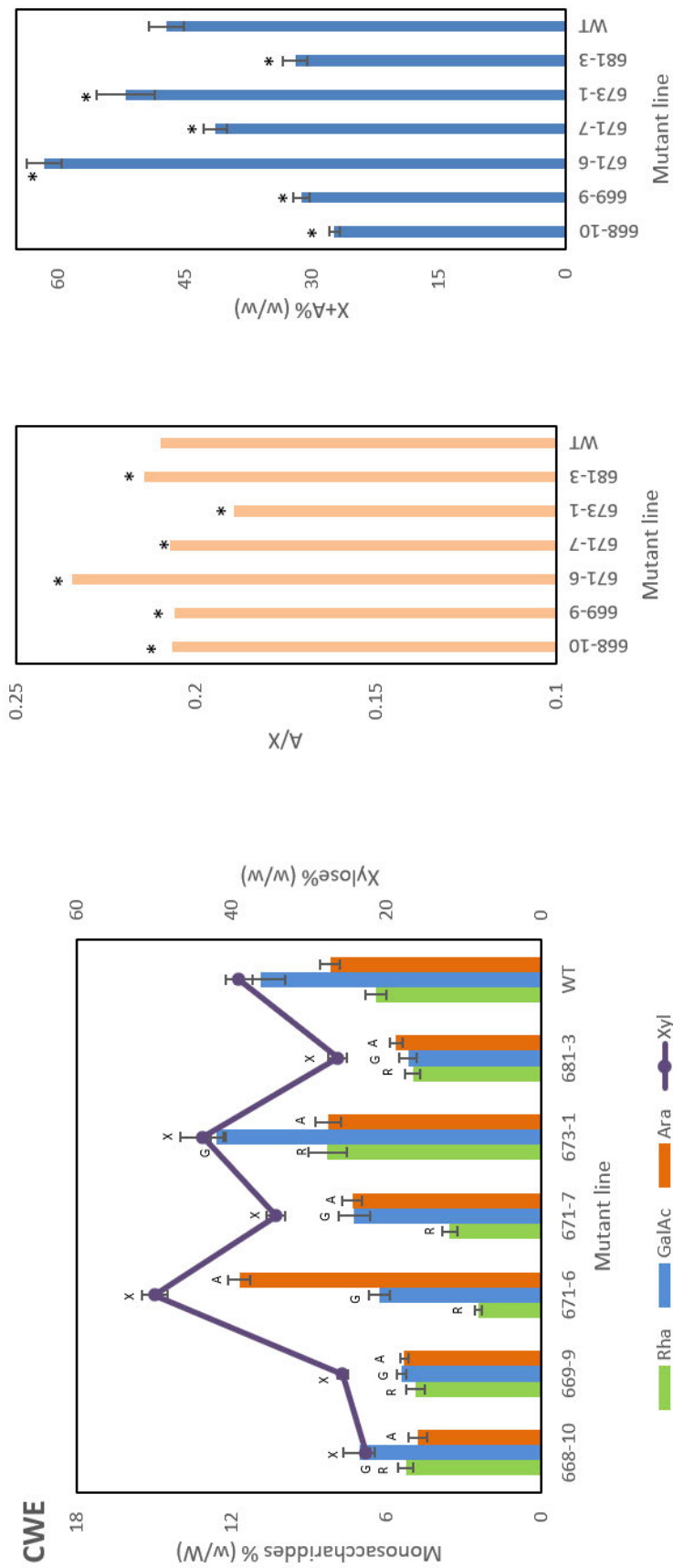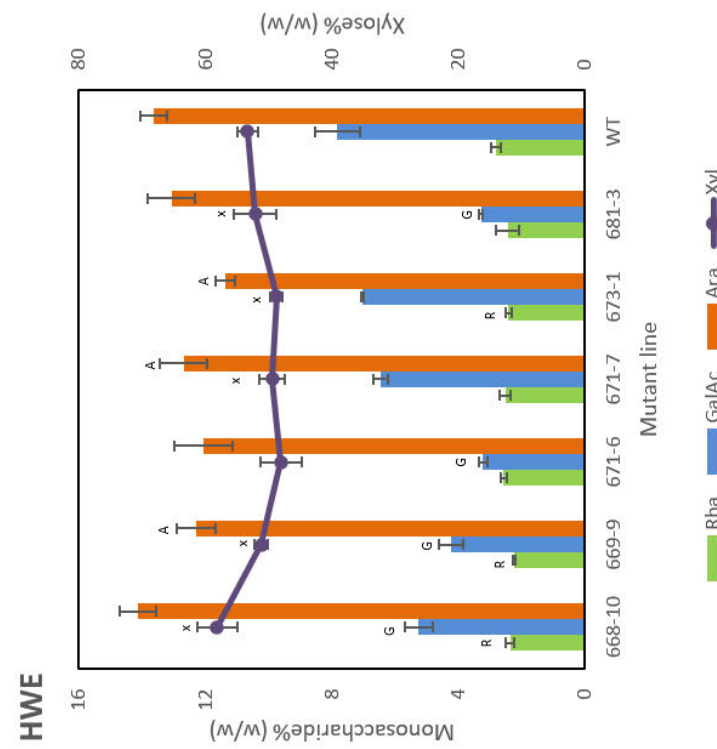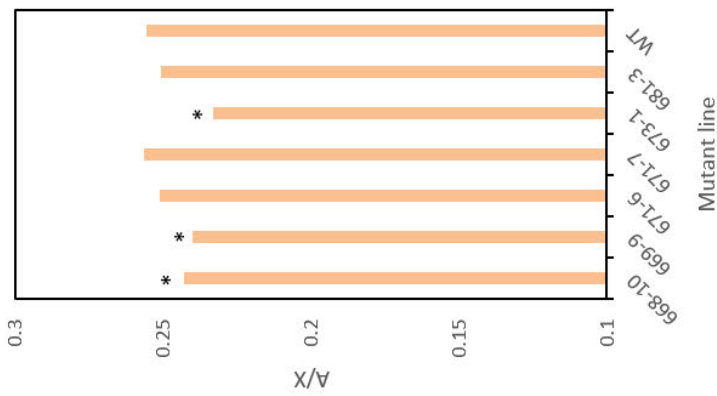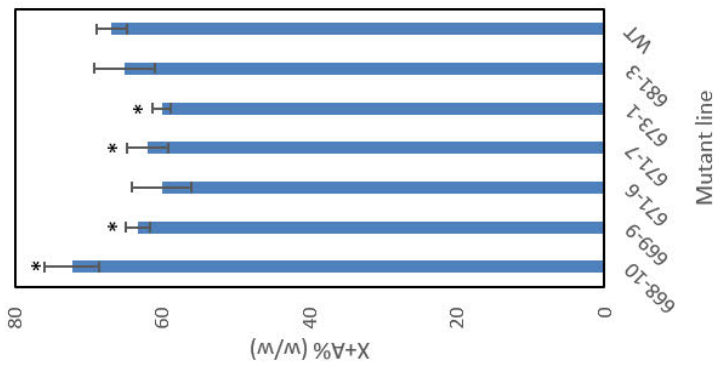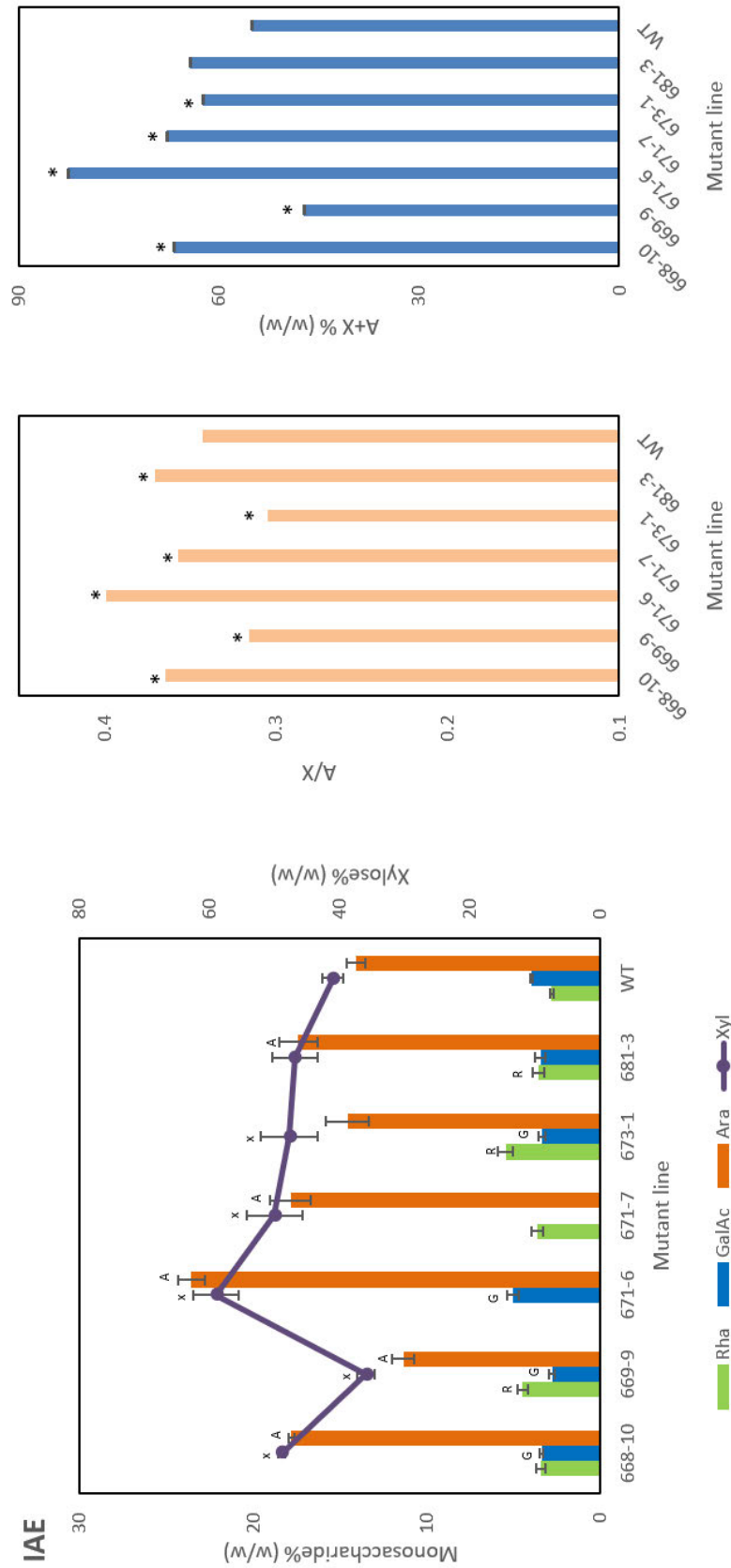
Figure 3-5. Monosaccharide composition of three mucilage fractions CWE, HWE and IAE of *P. ovata* mutants. Values which are significantly different (P<0.05) are denoted by different letters. R=Rhamnose, G=Galacturonic acid, A=Arabinose and X=Xylose. A/X is the arabinose/xylose ratio. A+X is the sum of arabinose and xylose. All values are mean and SD of at least three biological replicates with two technical replicates. Values significantly different from WT are indicated by * (P<0.05).
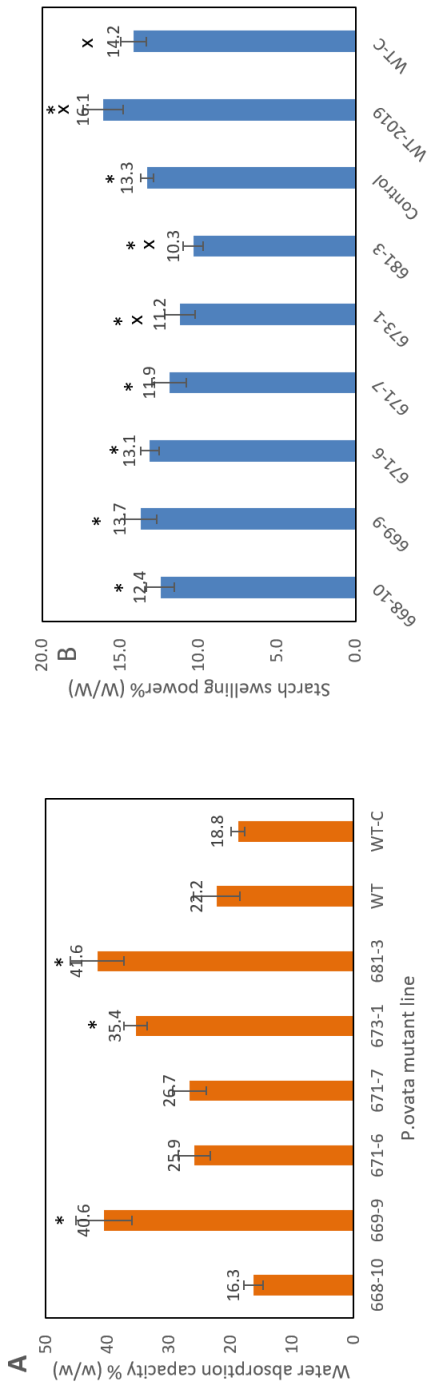
Figure 3-6. A. Water absorption capacity of seeds from six selected *P. ovata* mutants. Values significantly different from WT are indicated by * (P<0.05). Values are mean and standard deviation of at least five replicates. **B.** Starch swelling power of *P. ovata* mutants calculated as the weight of swollen starch in the presence of mucilage in comparison to the dry weight of starch. Values are mean and standard deviation of at least three replicates. Values significantly different from WT are indicated by * (P<0.05). Values significantly different from control are indicated by × (P>0.05)
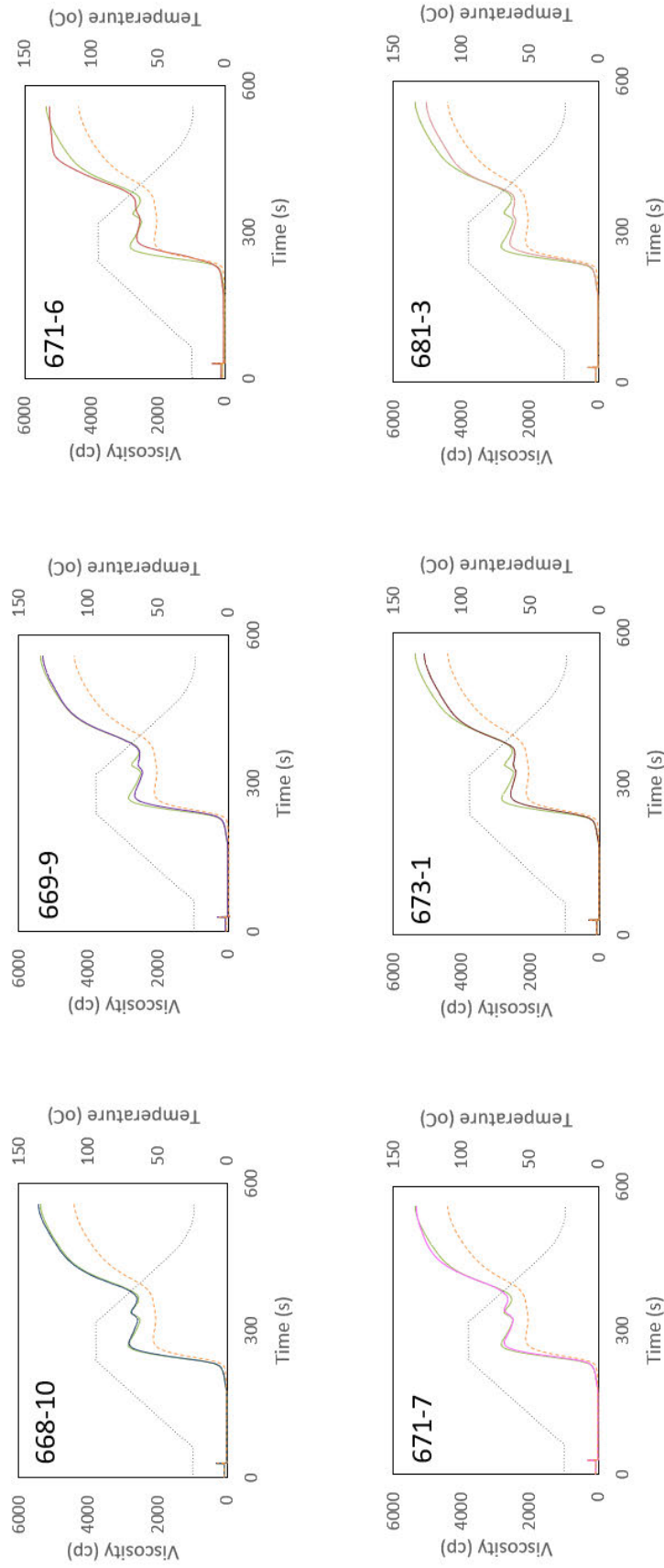
Figure 3-7. Change of RVA profile of rice flour with the addition of 1% P.ovata mutant husk. **B**. Change of RVA profile of rice starch with the addition of 1% *P. ovata* mutant husk.

Table 3-2. Pasting properties of rice flour upon addition of *P. ovata* mutant husk.

Pasting parameter	668-10	669-9	671-6	671-7	673-1	681-3	WT-GH	Control
Pasting Temperature (oC)	87.63±1.22	87.78±0.62	88.12±0.58	88.03±0.60	88.33±0.38	87.55±1.70	88.60±0.26	90.00±0.48*
Peak Viscosity (cP)	2730±252	2685±52	2648±49	2762±45	2595±45*	2670±43	2861±106	2129±57*
Trough Viscosity (cP)	2494±142	2462±33	2524±57	2492±43	2444±53	2456±18	2509±22	2056±51*
Breakdown Viscosity (cP)	236±116	223±23	124±10*	270±9	151±16	214±25	353±84	73±9*
Final Viscosity (cP)	5301±275	5316±115	5274±207	5320±274	5117±273	5229±178	5373±151	4412±79*
Setback Viscosity (cP)	2807±133	2854±82	2749±172	2828±233	2673±221	2774±161	2864±173	2356±100*

Values are means ± SD. Values which are significantly different from WT are indicated by * ($p < 0.05$)
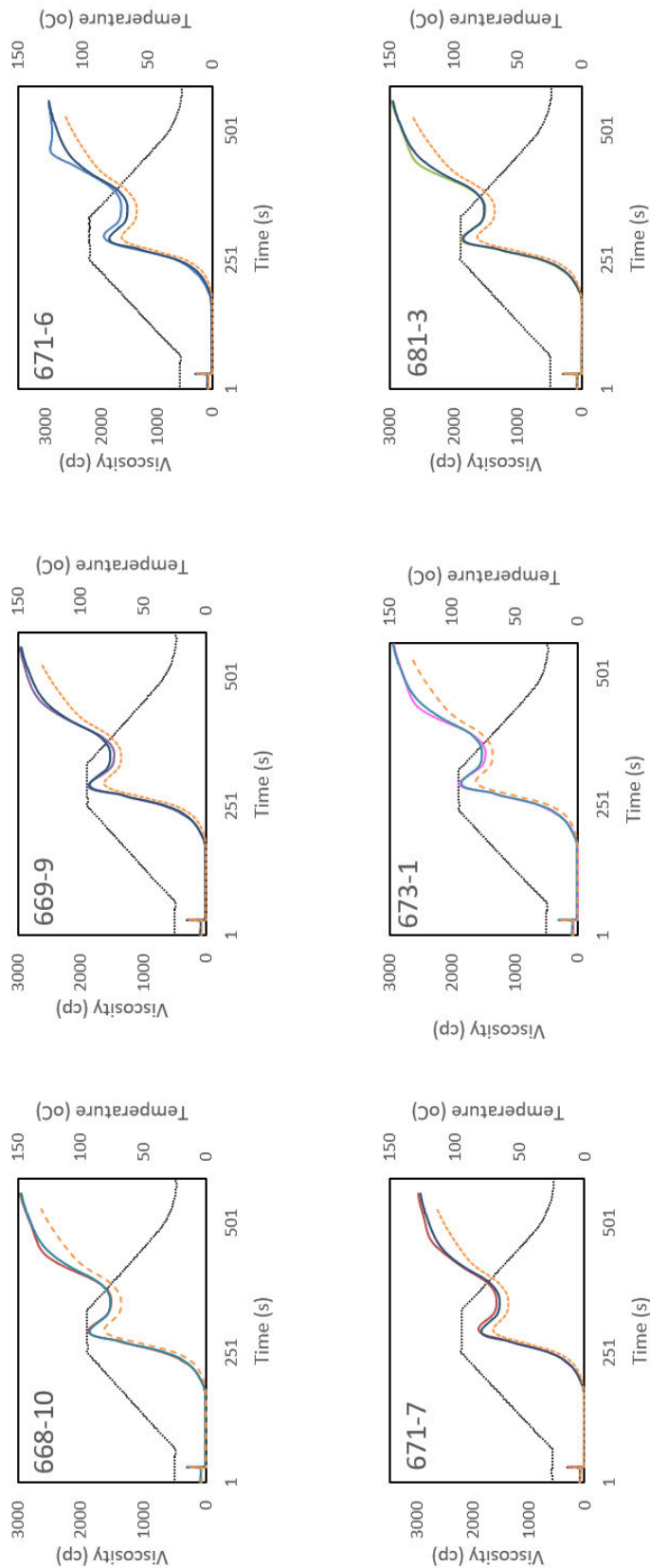
Figure 3-8. Change of RVA profile of rice starch with the addition of 1% *P. ovata* mutant husk.

Table 3-3. Pasting properties of rice starch upon addition of *P. ovata* mutant husk.

Pasting parameter	668-10	669-9	671-6	671-7	673-1	681-3	WT-GH	Control
Pasting Temperature (oC)	87.15±0.78	87.18±0.11	85.92±1.84	87.53±0.28	87.12±0.03	86.98±1.65	88.05±0.64	89.57±0.06
Peak Viscosity (cP)	1633±232	1454±11	1606±32*	1475±35*	1478±19	1477±27	1495±46	1637±27*
Trough Viscosity (cP)	1228±72	681±4	1247±20	1042±32*	1054±29	1057±55	1120±33	1323±37*
Breakdown Viscosity (cP)	405±72	432±4	359±20	433±32*	424±29	420±55	374±33	314±32
Final Viscosity (cP)	2971±22	2942±2	2955±22	2983±21	2933±41	2965±35	2956±45	2736±216
Setback Viscosity (cP)	1492±51	1488±9	1349±11	1508±43*	1455±27	1488±32	1461±53	1313±84

Values are means ± SD. Values which are significantly different from WT are indicated by with * ($p < 0.05$)

Table 3-4. Correlations of monosaccharide composition of mutant mucilage fractions and rice flour pasting properties.

| | | | Rice flour pasting | | | | | |
			Pasting T	Pasting V	Trough V	Break down V	Final V	Set back V
CWE	Rhamnose	Pearson R Sig.						
	Galacturonic acid	Pearson R Sig.	.803* 0.03					
	Xylose	Pearson R Sig.						
	Arabinose	Pearson R Sig.						
	X+A	Pearson R Sig.						
	A/X	Pearson R Sig.						
HWE	Rhamnose	Pearson R Sig.						
	Galacturonic acid	Pearson R Sig.						
	Xylose	Pearson R Sig.						
	Arabinose	Pearson R Sig.						
	X+A	Pearson R Sig.						
	A/X	Pearson R Sig.						
IAE	Rhamnose	Pearson R Sig.			-.867* 0.011			
	Galacturonic acid	Pearson R Sig.						
	Xylose	Pearson R Sig.						
	Arabinose	Pearson R Sig.						
	X+A	Pearson R Sig.						
	A/X	Pearson R Sig.						

Significant positive correlations are indicated in green. Significant negative correlations are indicated in pink ($P<0.05$)

Table 3-5. Correlations of monosaccharide composition of mutant mucilage fractions and rice starch pasting properties.

			Rice starch pasting					
			Pasting T	Pasting V	Trough V	Break down V	Final V	Set back V
CWE	Rhamnose	Pearson R	.786*					.755*
		Sig.	0.036					0.05
	Galacturonic acid	Pearson R						
		Sig.						
	Xylose	Pearson R						
		Sig.						
	Arabinose	Pearson R						-.812*
		Sig.						0.027
	X+A	Pearson R						
		Sig.						
	A/X	Pearson R				-.764*		-.875**
		Sig.				0.045		0.01
HWE	Rhamnose	Pearson R				-.781*		
		Sig.				0.038		
	Galacturonic acid	Pearson R	.815*					
		Sig.	0.025					
	Xylose	Pearson R						
		Sig.						
	Arabinose	Pearson R						
		Sig.						
	X+A	Pearson R						
		Sig.						
	A/X	Pearson R						
		Sig.						
IAE	Rhamnose	Pearson R				.900**		.935**
		Sig.				0.006		0.002
	Galacturonic acid	Pearson R						
		Sig.						
	Xylose	Pearson R			.780*			
		Sig.			0.039			
	Arabinose	Pearson R	-.798*		.772*			-.779*
		Sig.	0.032		0.042			0.039
	X+A	Pearson R			.787*			
		Sig.			0.036			
	A/X	Pearson R						
		Sig.						

Significant positive correlations are indicated in green. Significant negative correlations are indicated in pink (P<0.05)
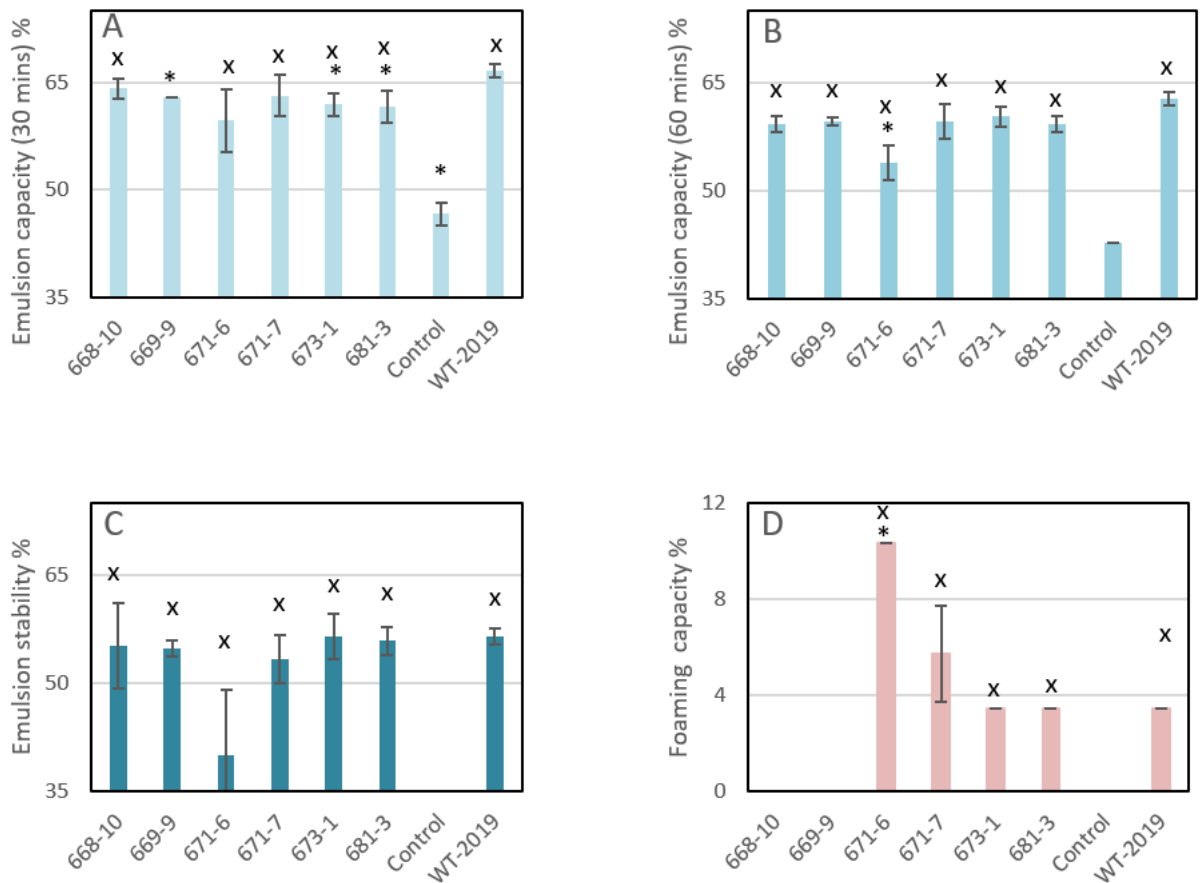
Figure 3-9. A. Emulsion capacity of mucilage from six selected *P. ovata* mutants at 30 minutes. **B.** Emulsion capacity of mucilage from six selected *P. ovata* mutants at 60 minutes. **C.** Emulsion stability of mucilage from six selected *P. ovata* mutants at 30 minutes. **D.** Foaming capacity of mucilage from six selected *P. ovata* mutants at 30 minutes. The values are mean and standard deviation of at least six replicates. Values significantly different from WT are indicated by * (P<0.05). Values significantly different from control are indicated by ˣ (P<0.05)

Table 3-6. Correlations of monosaccharide composition of mutant mucilage fractions and functional properties.

				Functional properties				
			WAC	SSP	EC30	EC60	ES30	FC
CWE	Rhamnose	Pearson R						
		Sig.						
	Galacturonic acid	Pearson R						
		Sig.						
	Xylose	Pearson R						.811*
		Sig.						0.027
	Arabinose	Pearson R					-.764*	.898**
		Sig.					0.046	0.006
	X+A	Pearson R						.829*
		Sig.						0.021
	A/X	Pearson R					-.821*	
		Sig.					0.023	
HWE	Rhamnose	Pearson R						
		Sig.						
	Galacturonic acid	Pearson R						
		Sig.						
	Xylose	Pearson R						
		Sig.						
	Arabinose	Pearson R						
		Sig.						
	X+A	Pearson R						
		Sig.						
	A/X	Pearson R						
		Sig.						
IAE	Rhamnose	Pearson R					.859*	
		Sig.					0.013	
	Galacturonic acid	Pearson R						
		Sig.						
	Xylose	Pearson R				-.757*		.786*
		Sig.				0.049		0.036
	Arabinose	Pearson R				-.805*	-.811*	.784*
		Sig.				0.029	0.027	0.037
	X+A	Pearson R				-.779*	-.771*	.792*
		Sig.				0.039	0.042	0.034
	A/X	Pearson R						
		Sig.						

Significant positive correlations are indicated in green. Significant negative correlations are indicated in pink (P<0.05)

Table 3-7. End-products from *in vitro* fermentation of mutant *P. ovata* substrates

	Acetate	Propionate	Butyrate	Branched-Chain	Total	AcTot	PrTot	BuTot	BrChPpn	NH₃	pH
Plantago			mmoles/ g DM				%			mmoles/ g DM	
M1	3.3a	1.8ab	0.83a	0.41b	6.4ab	53.008b	27.106a	12.756a	0.071bc	2.17a	6.41bc
M2	3.6a	1.9a	0.76ab	0.44b	6.7a	53.305b	27.831a	11.344b	0.075b	2.31a	6.39c
M3	3.4a	1.8ab	0.75ab	0.48a	6.5ab	53.120b	27.213a	11.355b	0.083a	2.15a	6.43ab
M4	3.5a	1.7b	0.80a	0.42b	6.4ab	55.217a	25.687b	12.578a	0.065c	2.08a	6.44ab
M5	3.3a	1.7b	0.68b	0.43b	6.1b	55.559a	25.730b	11.046b	0.077b	2.15a	6.46a
MSD	0.27	0.19	0.0889	0.043	0.49	1.1007	1.1756	0.9648	0.0064	0.291	0.039
Prob. Plantago	0.0689	0.0021	0.0001	0.0003	0.0215	<.0001	<.0001	<.0001	<.0001	0.2458	<.0001
Fraction											
W	3.7a	1.9a	0.79a	0.44a	6.8a	54.806a	26.633a	11.448b	0.071b	2.34a	6.42a
F	3.2b	1.7b	0.75b	0.43a	6.0b	53.272b	26.806a	12.168a	0.078a	2.01b	6.43a
MSD	0.12	0.08	0.04	0.020	0.22	0.4967	0.5305	0.4354	0.0029	0.1315	0.017
Prob. Fraction	<.0001	<.0001	0.054	0.2331	<.0001	<.0001	0.4856	0.0017	<.0001	<.0001	0.4477
Removal											
0	0.6f	0.2f	0.10e	0.12e	1.0f	56.382b	23.140c	9.286c	0.112a	1.32c	6.42ab
4	1.7e	0.7e	0.35d	0.18d	2.9e	58.004a	24.031c	11.936b	0.090b	1.98b	6.46a
10	2.6d	1.2d	0.68c	0.44c	4.9d	52.775c	24.489c	13.743a	0.060d	2.13b	6.43ab
24	3.5c	1.8c	0.87b	0.49b	6.7c	52.459c	26.744b	13.209a	0.076c	3.02a	6.41b
48	4.6b	2.6b	0.96b	0.56a	8.6983b	53.003c	29.339a	11.206b	0.065d	2.21b	6.45ab
72	5.1a	2.8a	1.12a	0.59a	9.6159a	53.032c	29.111a	11.677b	0.062d	2.25b	6.41b
MSD	0.32	0.23	0.107	0.052	0.5989	1.3342	1.425	1.1695	0.0078	0.353	0.047
Prob. Removal	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0052
Prob. Plantago x FX* Removal	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0017	<.0001

a,b,c,d Superscripts which differ within the same column are significantly different from each other (P < 0.05).

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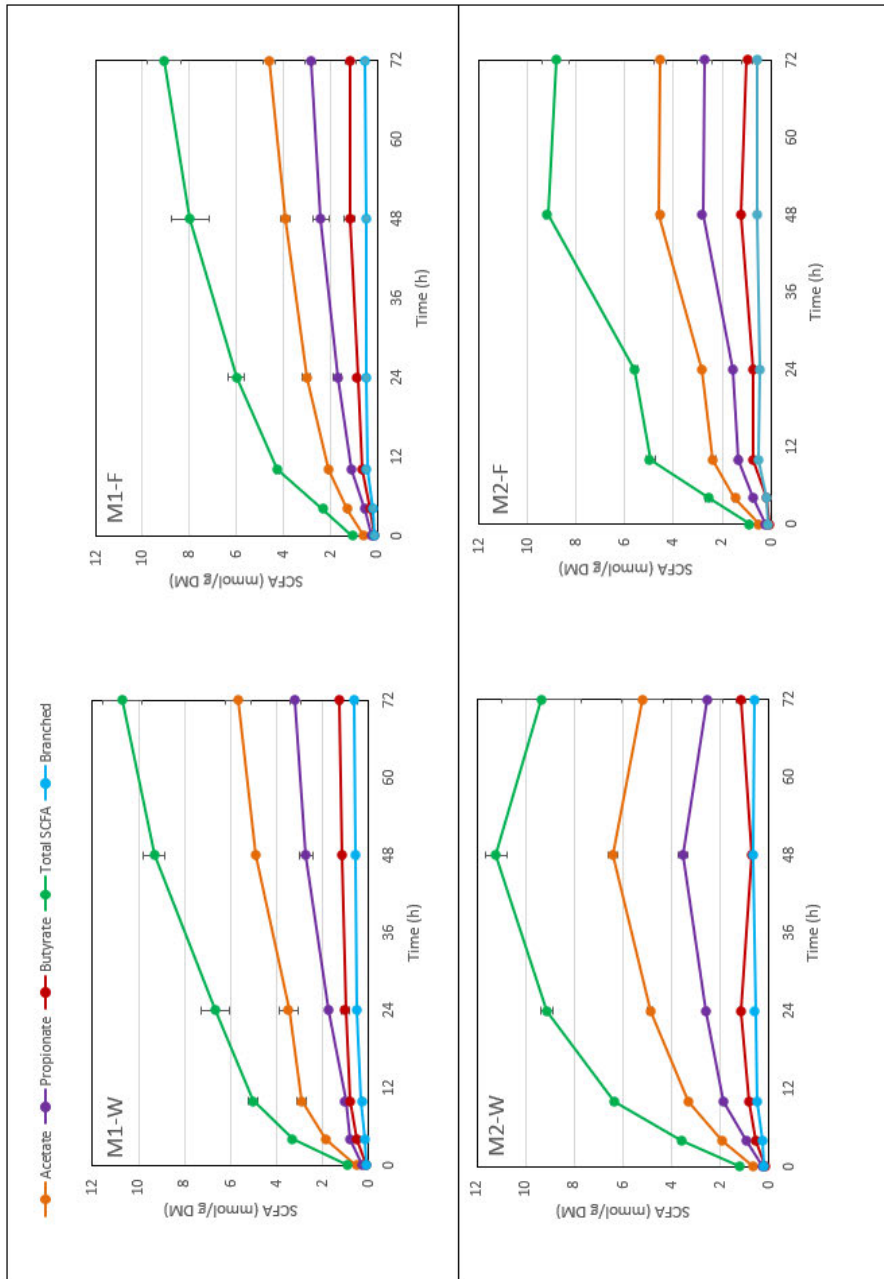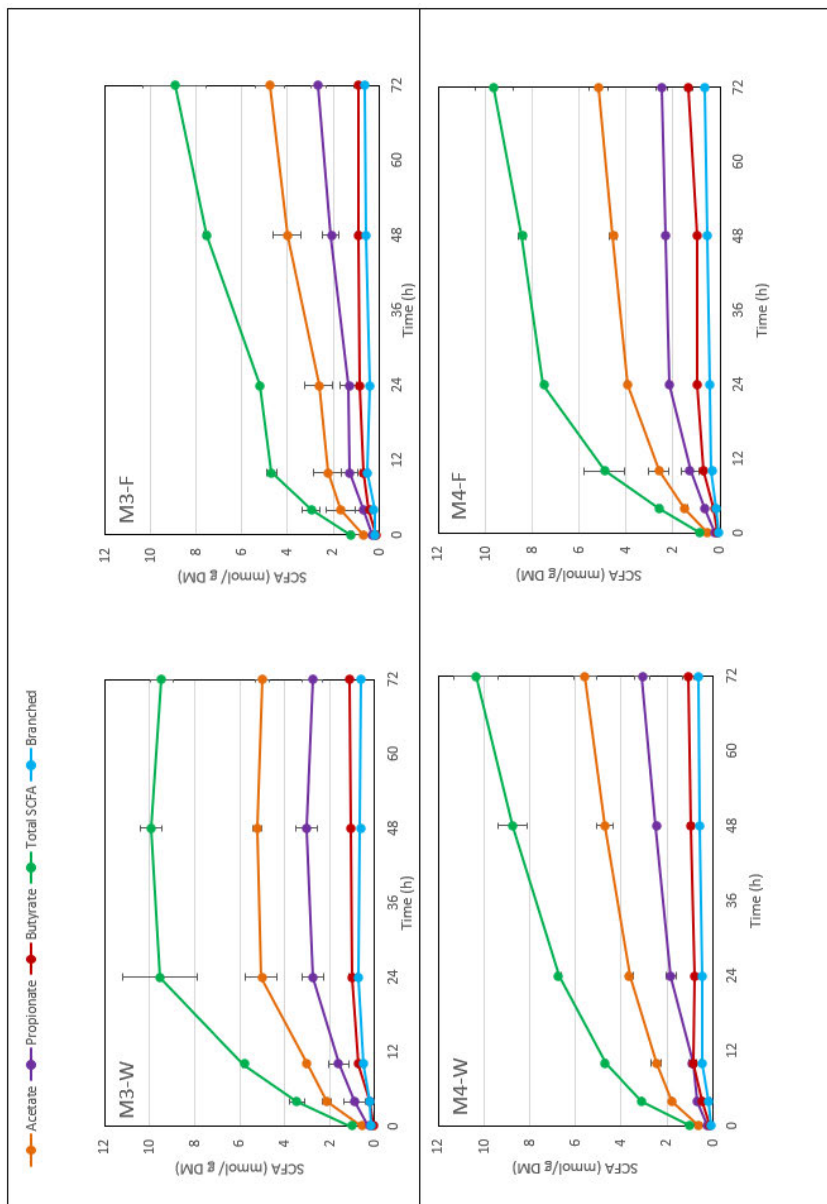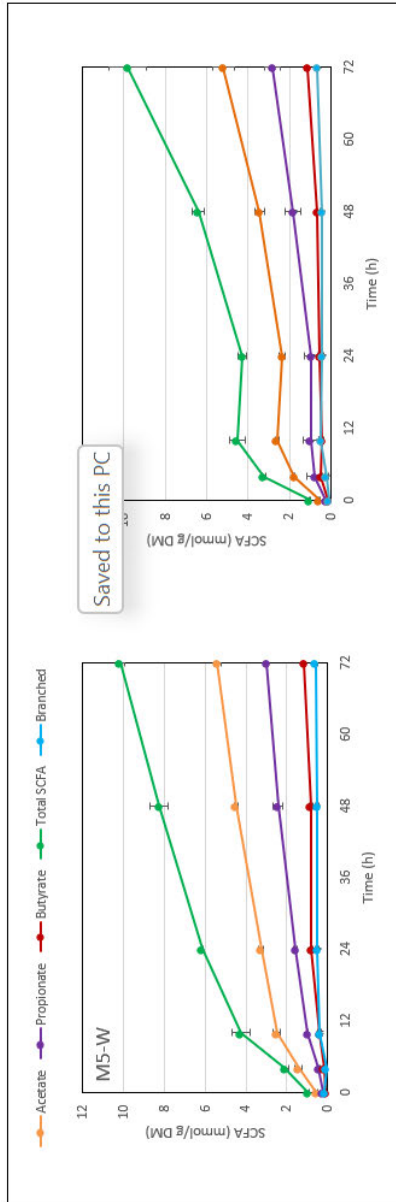
Figure 3-10. SCFA production in time, of mutant *P. ovata*; Total SCFA, acetate, propionate, butyrate, branched chain SCFA. Data are expressed as means while the error bars indicate standard deviation.
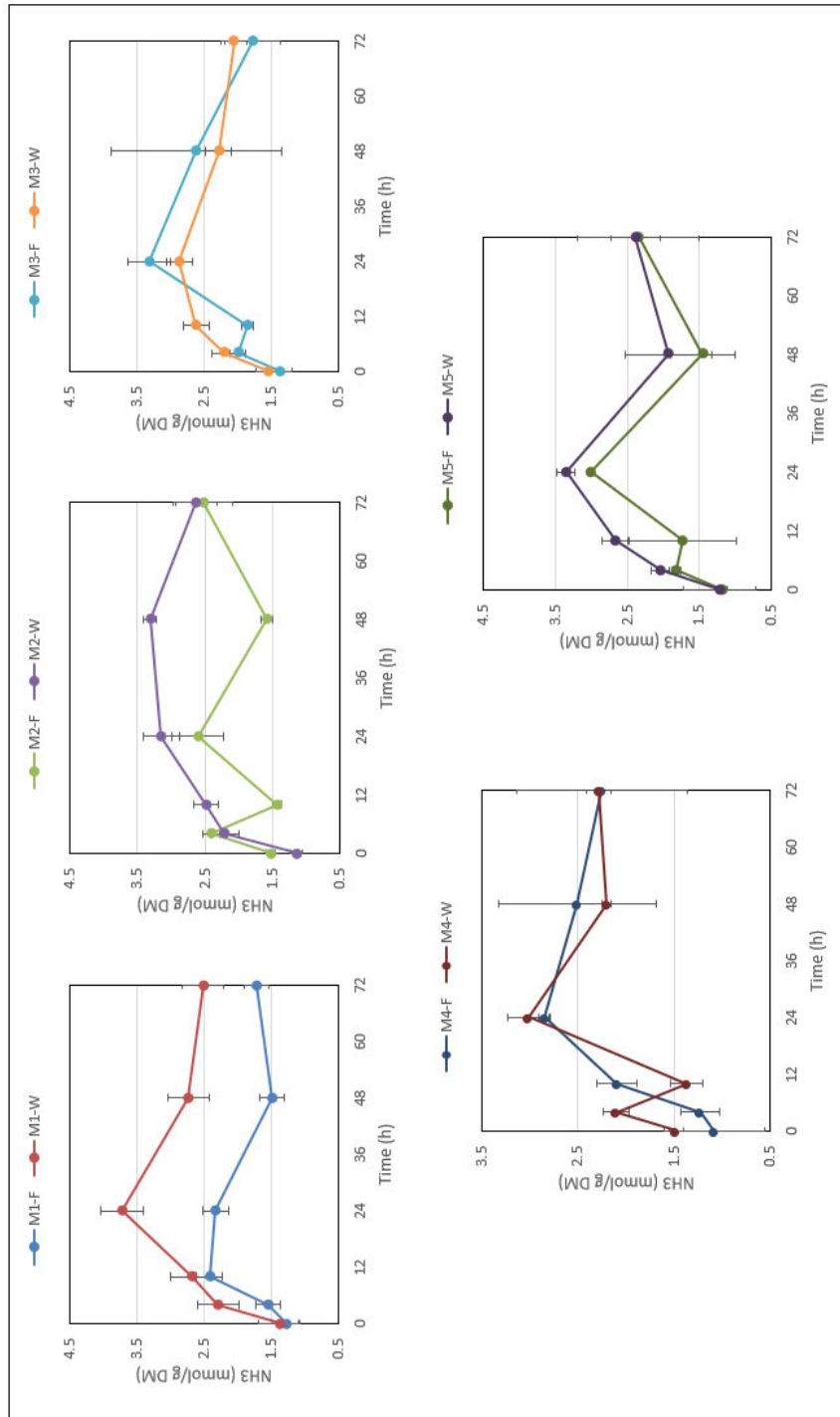
Figure 3-11. NH₃ changes of *P. ovata*. Data are expressed as the mean, and the error bars show the standard deviation.

Table 3-8. Correlations between the monosaccharide composition and fermentation products of mutant *P. ovata* .

		CWE- Arabinose	HWE- A/X	IAE – Galacturonic acid
Acetate	Pearson R			
	Sig.			
Propionate	Pearson R			.912*
	Sig.			0.031
Butyrate	Pearson R	.957*		
	Sig.	0.011		
Total SCFA	Pearson R			
	Sig.			
Branched	Pearson R			
	Sig.			
NH3	Pearson R		.904*	
	Sig.		0.035	
pH	Pearson R			
	Sig.			

CWE = cold water extractable fraction, HWE = Hot water extractable fraction, IAE = Intense agitation extractable fraction. Significant correlations (p <0.05) positive is highlighted in green and negative in pink. * Correlation is significant at the 0.05 level (2-tailed).

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Supplementary figures

Supplementary Table 3-1. Monosaccharide analysis of fractions extracted from *P. ovata* mutant seed husk. Values are mean ± standard deviation in % mol/mol. nd= not detected. Values which are significantly different from WT are indicated by with * (p<0.05)

	D-Man	D-Rha	D-GalAc	Xyl	Ara	X+A	A/X	Unaccounted
Cold water extractable mucilage (CWE) fraction								
668-10	nd	5.3±0.3*	7.1±0.6*	22.7±0.6*	4.8±0.4*	27.4±0.6*	0.207*	60.2
669-9	nd	4.9±0.4*	5.4±0.2*	25.7±0.2*	5.3±0.2*	31.3±0.9*	0.206*	58.6
671-6	nd	2.4±0.1*	6.3±0.4*	50.0±1.7*	11.7±0.4*	61.7±2.1*	0.234*	29.6
671-7	nd	3.6±0.3*	7.3±0.6*	34.3±1.2*	7.3±0.4*	41.4±1.4*	0.207*	47.5
673-1	nd	8.3±0.7*	12.6±0.3*	43.8±3.0*	8.3±0.5*	52.1±3.4*	0.189*	27.1
681-3	nd	5.0±0.3*	5.2±0.3*	26.4±1.2*	5.6±0.3*	32.0±1.4*	0.214*	57.8
WT	nd	6.4±0.4*	10.9±0.9*	39.1±1.7*	8.2±0.4*	47.3±2.1*	0.210*	35.4
Hot water extractable mucilage (HWE) fraction								
668-10	nd	2.4±0.1*	5.3±0.5*	58.1±3.2*	14.1±0.6	72.3±3.7	0.243*	20.1
669-9	nd	2.2*	4.2±0.4*	51.1±1.1*	12.3±0.6*	63.4±1.7*	0.240*	30.1
671-6	nd	2.6±0.1	3.2±0.1	48.0±3.2	12.1±0.9	60.1±4.1	0.251	34.1
671-7	nd	2.5±0.2	6.4±0.2	49.4±2.1*	12.7±0.8*	62.1±2.8*	0.257	28.9
673-1	nd	2.4±0.1*	7.0	48.8±1.0*	11.4±0.3*	60.1±1.3*	0.233*	30.4
681-3	nd	2.4±0.4	3.3±0.1	52.1±3.4*	13.1±0.8	65.2±4.1*	0.251	29.1
WT	nd	2.8±0.2	7.8±0.7	53.3±1.6	13.6±0.4	66.9±2.0	0.256	22.4
Intense Agitation extraction mucilage (IAE) fraction								
668-10	nd	3.4±0.3*	3.4±0.1*	48.9±0.5*	17.8±0.2*	66.7±0.7*	0.364*	26.4
669-9	nd	4.5±0.3*	2.8±0.2*	36.0±1.3*	11.4±0.6*	47.3±1.9*	0.316*	45.4
671-6	nd	0.0*	5.0±0.3*	59.1±3.5*	23.6±0.8*	82.6±4.43*	0.400*	12.3
671-7	nd	3.6±0.3	0.0*	50.1±4.3*	17.8±1.2*	67.9±5.5*	0.357*	28.4
673-1	nd	5.5±0.4	3.3±0.2*	47.8±4.4*	14.6±1.2	62.4±5.7*	0.305*	28.8
681-3	nd	3.6±0.3*	3.5±0.3	47.0±3.5	17.4±1.1*	64.4±4.6	0.371*	28.6
WT	nd	2.8±0.1	4.0±0.1	41.1±1.6	14.1±0.6	55.2±2.1	0.343	38.0

Supplementary Table 3-2. End products of fermentation of all *P. ovata* substrates. Values are mean and standard deviation for 0 h, 4 h, 10 h, 24 h, 48 h and 72 h.

Level of Plantago	Level of Fx	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
M1	F	0	2	0.6026 1508	0.0213 0439	0.2351 8200	0.0125 3872	0.1062 0329	0.0086 2964	1.0751 716	0.0371 7254	56.047 5144	0.0437 2314	21.866 8131	0.4101 9212	9.8698 234	0.4613 9422	0.1221 5849	0.0091 5309	0.1311 7122	0.0053 0021	1.2855 7422	0.0068 7450	6.3650 0000	0.0070 7107
M1	F	4	2	1.2790 1561	0.0082 2962	0.5297 1840	0.0673 6792	0.2917 6433	0.0047 7431	2.2781 307	0.0381 5482	56.154 1073	1.3017 3050	23.230 8215	2.5680 8102	12.807 2229	0.0049 2793	0.0780 7848	0.0126 1423	0.1776 3234	0.0257 5778	1.5482 3842	0.1868 4915	6.4250 0000	0.0212 1320
M1	F	10	2	2.0692 5556	0.0111 4615	1.1114 8293	0.0276 1060	0.6370 0973	0.0126 8411	4.2496 559	0.0773 4913	48.697 9877	0.6240 8171	26.153 0763	0.1736 9464	14.989 4445	0.0256 4694	0.1015 9492	0.0042 4740	0.4319 0770	0.0259 0827	2.4177 4240	0.2780 6664	6.3950 0000	0.0212 1320
M1	F	24	2	2.9943 3198	0.1723 3939	1.6941 0425	0.1738 7813	0.8367 1559	0.0261 5866	5.9984 565	0.3369 8505	49.916 4415	0.0688 2505	28.205 4221	1.3141 7265	13.983 1633	1.2216 4471	0.0789 4973	0.0016 1353	0.4733 0466	0.0169 2619	2.3284 4130	0.2892 8397	6.3700 0000	0.0141 4214
M1	F	48	2	3.8999 8739	0.2192 5274	2.4100 5507	0.3479 2043	1.1801 4220	0.2365 0475	7.9802 902	0.8034 6499	48.980 1865	2.1839 5451	30.133 3470	1.3258 8581	14.713 5952	1.4822 2884	0.0617 2871	0.0062 4160	0.4901 0559	0.0002 1293	1.4921 1902	0.1469 5535	6.3900 0000	0.0282 8427
M1	F	72	5	4.5966 3713	0.2691 2984	2.8419 2021	0.2514 0530	1.1311 1389	0.2379 4935	9.0924 037	0.7341 9063	50.632 4039	1.3172 7234	31.251 6197	0.8156 2743	12.351 0823	1.8463 4280	0.0576 4894	0.0039 0756	0.5227 3249	0.0348 8426	1.7220 6151	0.2334 7400	6.4620 0000	0.1522 1695
M1	W	0	2	0.5314 2631	0.0007 5300	0.2346 9117	0.0010 2930	0.0619 8343	0.0040 6856	0.9229 080	0.0095 5556	57.585 2296	0.6778 1345	25.430 3159	0.1517 7127	6.7141 785	0.3713 2389	0.1027 0276	0.0045 8261	0.0948 0709	0.0052 1071	1.3797 1370	0.0793 1251	6.4000 0000	0.0424 2641
M1	W	4	2	1.8448 4402	0.0448 8517	0.7870 5164	0.0111 8126	0.4786 2194	0.0057 3622	3.2734 418	0.0243 2797	56.354 3908	0.9523 7053	24.042 9456	0.1628 8960	14.622 4222	0.2839 0777	0.0498 0241	0.0083 1352	0.1629 2418	0.0260 0224	2.2952 5845	0.2868 5454	6.3900 0000	0.0141 4214
M1	W	10	2	2.9120 0016	0.2190 0130	0.9841 6469	0.0830 0366	0.7952 0947	0.0066 9387	4.9958 528	0.2028 9977	58.247 3708	2.0180 2430	19.749 6607	2.4635 5695	15.927 8075	0.5128 9762	0.0607 5161	0.0095 8430	0.3044 7843	0.0602 0825	2.6918 3566	0.2275 2332	6.3700 0000	0.0424 2641
M1	W	24	2	3.4602 6736	0.3977 6163	1.7383 2279	0.0127 3022	0.9900 4959	0.1964 0622	6.6734 532	0.6195 4612	51.797 7718	1.1515 8596	26.152 1703	2.2371 4066	14.762 6448	1.5725 7159	0.0728 7413	0.0048 7017	0.4848 1345	0.0126 4804	3.7231 8019	0.0282 8841	6.3750 0000	0.0353 5534
M1	W	48	2	4.8827 6979	0.0718 5764	2.7159 3686	0.3023 0294	1.1631 3306	0.0915 5156	9.3028 690	0.4715 7396	52.534 6283	1.8906 2095	29.149 7052	1.7719 3205	12.494 0593	0.3507 8240	0.0582 1607	0.0023 2094	0.5410 2925	0.0058 6183	2.7375 5960	0.4089 7252	6.3850 0000	0.0070 7107
M1	W	72	5	5.6614 2223	0.5708 0011	3.1761 8202	0.2382 4194	1.2588 2134	0.0676 8343	10.692 3415	0.8666 8792	52.889 0791	1.6017 6212	29.735 3694	1.1336 1360	11.828 5013	1.0535 7232	0.0554 7050	0.0057 9293	0.5959 1597	0.1016 8269	2.5167 2815	0.3458 6757	6.4300 0000	0.0353 5534
M2	F	0	2	0.5227 7089	0.0098 3718	0.2285 1834	0.0104 8634	0.0565 5132	0.0018 0379	0.9151 704	0.0044 1092	57.126 0464	1.3502 3698	24.967 5597	1.0254 9644	6.1789 176	0.1673 1784	0.1172 7476	0.0015 7423	0.1073 2986	0.0019 5798	1.5209 4427	0.0639 2900	6.4100 0000	0.0141 4214
M2	F	4	2	1.4831 1552	0.0428 7630	0.7094 6089	0.0619 2849	0.2009 4502	0.0001 0738	2.5618 547	0.1282 6593	57.922 9596	1.2264 2085	27.667 4153	1.0320 8910	7.8534 708	0.3890 1282	0.0655 6154	0.0058 3345	0.1683 3326	0.0233 5375	2.3990 3370	0.1218 1669	6.3900 0000	0.0141 4214

Level of Plantago	Level of Fx	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
M2	F	10	2	2.37992826	0.11763221	1.32009675	0.07814074	0.74612165	0.00885564	4.9586349	0.20342924	47.9873554	0.40357676	26.6122510	0.48407752	15.0632559	0.79656417	0.10337138	0.00091090	0.51248827	0.01651193	1.43420527	0.06355309	6.33000000	0.02828427
M2	F	24	2	2.86301540	0.06092366	1.54083724	0.07331350	0.71199830	0.07539791	5.5902037	0.11574400	51.2371321	2.15068310	27.5554977	0.74093306	12.7253007	1.08527591	0.08482069	0.00324474	0.47435274	0.02795625	2.59624087	0.38243983	6.32500000	0.00707107
M2	F	48	2	4.58865907	0.10840623	2.80774749	0.03725522	1.21333848	0.00478255	9.1735361	0.12673229	50.0172241	0.49074054	30.6071515	0.01672119	13.2281304	0.23488066	0.06147494	0.00239139	0.56379105	0.01414661	1.57429419	0.08417606	6.61500000	0.00707107
M2	F	72	5	4.57851698	0.24754210	2.72429339	0.30328865	0.98542907	0.22897943	8.8371538	0.56617040	51.8501184	1.51269677	30.8009523	2.48691070	11.1543030	2.48543209	0.06194626	0.00540604	0.54891433	0.07733628	2.51475940	0.41699066	6.35400000	0.03646917
M2	W	0	2	0.60889409	0.00713270	0.26303958	0.01022790	0.14004291	0.01199937	1.1824645	0.02535953	51.5119625	1.70794971	22.2408701	0.38797944	11.8351481	0.76095581	0.14412019	0.00559014	0.17048789	0.01026497	1.14595299	0.08484719	6.35500000	0.00707107
M2	W	4	2	1.91338021	0.04759113	0.91469770	0.06771259	0.51574470	0.02002413	3.5798182	0.11440149	53.4551425	0.37885587	25.5343275	1.07549976	14.4054263	0.09900244	0.06605104	0.00795646	0.23599558	0.02092635	2.20521702	0.21582633	6.38000000	0.01414214
M2	W	10	2	3.29816715	0.00155100	1.83611571	0.01346919	0.79551612	0.06640120	6.3694137	0.09330563	51.7867088	0.73427345	28.8286201	0.21084412	12.4833323	0.85963244	0.06901339	0.00085485	0.43961469	0.01188424	2.48178170	0.19029139	6.36500000	0.00707107
M2	W	24	2	4.85004652	0.11599282	2.56343898	0.06069442	1.15368104	0.05611426	9.1074155	0.24530743	53.2559848	0.16083666	28.1479582	0.09173418	12.6637870	0.27504018	0.05932270	0.00022469	0.54024892	0.01250592	3.14522741	0.26294524	6.42000000	0.05656854
M2	W	48	2	6.38389550	0.19987113	3.50747179	0.21138182	0.68980381	0.04714124	11.2090612	0.43523258	56.9613109	0.42860905	31.2783633	0.67131575	6.1504552	0.18174985	0.05609871	0.00424457	0.62789015	0.02316161	3.31302411	0.09514918	6.39000000	0.02828427
M2	W	72	5	5.17264543	0.87276305	2.51463713	0.64973605	1.10540072	0.19151640	9.3379201	1.64924949	55.4734696	2.42972804	26.8101698	3.76611509	11.8719902	0.98672713	0.05844370	0.00653618	0.54523682	0.10584939	2.64379216	0.32858944	6.36800000	0.02167948
M3	F	0	2	0.65061268	0.01943884	0.27834310	0.01781894	0.11798082	0.01457913	1.2347896	0.09071460	52.7747578	2.30286763	22.5495881	0.21354485	9.6240729	1.88773645	0.15051581	0.00404149	0.18785296	0.06803595	1.37224294	0.18466902	6.42500000	0.02121320
M3	F	4	2	1.67208765	0.16534054	0.65859277	0.17044836	0.43279533	0.02771257	2.9627808	0.37244953	56.5323480	1.52606399	22.0414291	2.98216987	14.6648218	0.90814980	0.06761401	0.00547956	0.19930507	0.00894807	1.99881333	0.12010544	6.50500000	0.00707107
M3	F	10	2	2.23756435	0.09635489	1.28804337	0.02119472	0.67156346	0.14473763	4.7016417	0.22208492	47.5958247	0.19882966	27.4368644	1.74679111	14.2267602	2.40643892	0.10740551	0.00460818	0.50447051	0.00218713	1.85653334	0.09011629	6.53000000	0.01414214
M3	F	24	2	2.62119331	0.00027869	1.31519928	0.00013412	0.85723806	0.00065404	5.2124696	0.00059134	50.2869759	0.00035836	25.2317883	0.00028945	16.4459094	0.01068195	0.08035326	0.00010034	0.41883891	0.00047551	3.32291448	0.31517294	6.43000000	0.01414214
M3	F	48	2	4.00806709	0.04047603	2.08932375	0.00485712	0.88953674	0.06638068	7.5536209	0.09390438	53.0623034	0.12380554	27.6624342	0.40819313	11.7717430	0.73245008	0.07503519	0.00200451	0.56669329	0.00809521	2.62281683	1.26770917	6.44000000	0.05656854

Level of Plantago	Level of Fx	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
M3	W	0	2	0.56259197	0.00681727	0.25359885	0.00420061	0.07018153	0.00890362	1.0452330	0.06041345	53.8957250	2.46289527	24.2913832	1.00213546	6.7010137	0.46451926	0.15111878	0.03000511	0.15886070	0.04049194	1.54242949	0.03915221	6.43500000	0.00707107
M3	W	4	2	2.12094339	0.19836255	0.90350965	0.11321284	0.24039872	0.01145494	3.4576034	0.32808951	61.3454176	0.08402726	26.0932185	0.79834844	6.9999879	0.99552098	0.05561376	0.00281200	0.19275162	0.02796907	2.20121048	0.18425921	6.43000000	0.01414214
M3	W	10	2	2.99889783	0.06053069	1.58971941	0.03435544	0.74671528	0.00826638	5.8200069	0.06107917	51.5247730	0.49930848	27.3131408	0.30365605	12.8301055	0.00738593	0.08331981	0.00810350	0.48467438	0.04207335	2.62058630	0.06853655	6.37500000	0.00707107
M3	W	24	2	5.02628821	0.70834437	2.74259749	0.81900177	1.03340199	0.08583631	9.5395489	1.67176023	52.8498555	1.83633941	28.4341101	3.60238865	10.9216831	1.01417845	0.07794351	0.00751871	0.73726120	0.05857778	2.86983992	0.05095274	6.40500000	0.04949747
M3	W	48	2	5.20594668	0.18283495	3.02178476	0.32978798	1.05509701	0.02117332	9.9289634	0.45679117	52.4450679	0.57135363	30.3897975	1.92336356	10.6426249	0.70287186	0.06522510	0.00649138	0.64613499	0.03465843	2.28770012	0.18078985	6.37000000	0.07071068
M3	W	72	5	4.97936559	0.30978132	2.76718234	0.22180070	1.11037055	0.02544376	9.4593068	0.52163133	52.6249694	0.56036684	29.2312587	1.13873869	11.7668248	0.70619800	0.06376947	0.00599897	0.60238831	0.05746082	2.05704758	0.27593516	6.40600000	0.02190890
M4	F	0	2	0.54174302	0.01100906	0.19857126	0.00342702	0.08651797	0.02558920	0.8678089	0.00560032	62.4319307	1.67150242	22.8836634	0.54258194	9.9603960	2.84443558	0.04724010	0.00670351	0.04097661	0.00555281	1.07958508	0.51979598	6.34500000	0.09192388
M4	F	4	2	1.50036122	0.10094361	0.65232731	0.06434499	0.25224759	0.05544298	2.5832443	0.09748124	58.0481026	1.71712753	25.2232118	1.53903722	9.8122412	2.51652842	0.06916444	0.00739636	0.17830815	0.01236438	1.22309934	0.20098528	6.42000000	0.01414214
M4	F	10	2	2.59265019	0.43850597	1.25761690	0.38169412	0.69790572	0.03174020	4.9081388	0.85378830	52.8459716	0.25848401	25.3299339	3.37052629	14.4949093	3.16812638	0.07329185	0.00056084	0.35996600	0.06532841	2.09044465	0.20609635	6.40000000	0.01414214
M4	F	24	2	3.95182008	0.06603781	2.16134918	0.05028369	0.98396648	0.05560559	7.5122920	0.05376136	52.6029201	0.50261307	28.7739674	0.87527173	13.0957728	0.64647547	0.05527340	0.00273817	0.41515629	0.01759834	2.84640282	0.06081617	6.37000000	0.00000000
M4	F	48	2	4.55182581	0.14751543	2.34195500	0.00098617	0.98562181	0.00204160	8.4382092	0.16040972	53.9361603	0.72286178	27.7592978	0.53938918	11.6823428	0.19788580	0.06622199	0.00014413	0.55880657	0.01183887	2.50761405	0.81867270	6.41500000	0.07778175
M4	F	72	5	5.16771627	0.42331165	2.47834842	0.23114341	1.32779373	0.17814339	9.6105017	0.80066722	53.7924763	1.33024190	25.7919492	1.20779978	13.7863432	1.08220951	0.06629231	0.00429835	0.63664326	0.06160064	2.25083346	0.88384950	6.36800000	0.07661593
M4	W	0	2	0.60948599	0.10136745	0.22007130	0.04607291	0.12237568	0.00908797	1.0088135	0.17416969	60.4496443	0.38832839	21.7446956	0.81286054	12.2352389	1.21153286	0.05570421	0.00787001	0.05688052	0.01764135	1.47813246	0.07619968	6.52500000	0.07778175
M4	W	4	2	1.78069597	0.01202002	0.69036127	0.06415053	0.45227709	0.01392908	3.0955800	0.03715094	57.5302958	1.07873365	22.2906859	1.80480961	14.6141676	0.62535547	0.05564851	0.00100720	0.17224570	0.00105049	2.10069613	0.12948285	6.53500000	0.00707107
M4	W	10	2	2.47114030	0.19948165	0.87152456	0.02181708	0.86684108	0.14971639	4.6801689	0.02369405	52.8117040	4.52964165	18.6207065	0.37188996	18.5137167	3.10522422	0.10053873	0.01052527	0.47066291	0.05164223	1.36624974	0.16500632	6.48500000	0.09192388

Level of Plantago	Level of Fx	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
M4	W	48	2	4.71062167	0.35780206	2.48834080	0.05705698	0.96384675	0.12317786	8.7421096	0.62240245	53.8751124	0.25716946	28.5128784	1.37733202	11.0030620	0.62564450	0.06608947	0.00494518	0.57930036	0.08436556	2.19624586	0.04798709	6.53000000	0.08485281
M4	W	72	4	5.56970255	0.50000932	3.08627524	0.31076770	1.05852639	0.24073773	10.3503078	0.94985144	53.8192793	0.55155128	29.8236926	1.55604772	10.2175263	2.06965505	0.06139502	0.00439453	0.63580366	0.08091457	2.28225684	0.12787710	6.50250000	0.02500000
M5	F	0	2	0.57626035	0.01820589	0.23638892	0.02785436	0.12890865	0.00034964	1.0367603	0.02652359	55.6234622	3.17905979	22.7738172	2.10404737	12.4374339	0.28446482	0.09165287	0.01359477	0.09520234	0.01652548	1.18412175	0.08110241	6.54500000	0.02121320
M5	F	4	2	1.76681583	0.04767343	0.75962404	0.00695926	0.48493883	0.00515641	3.2295492	0.07433882	54.7053192	0.21693879	23.5248070	0.32601475	15.0178226	0.18602181	0.06752051	0.00295098	0.21817050	0.01454972	1.82442468	0.06258793	6.68000000	0.14142136
M5	F	10	2	2.59962527	0.07927845	0.96448612	0.09496937	0.45021490	0.12781983	4.5162760	0.37265095	57.6852081	3.00437851	21.3416795	0.34186121	9.8856083	2.01451409	0.11087504	0.00648003	0.50194968	0.07058330	1.73601693	0.74448553	6.60000000	0.02828427
M5	F	24	2	2.36584536	0.14273063	0.92808761	0.00919189	0.54093315	0.08966583	4.2557196	0.20877207	55.5767440	0.62743587	21.8289749	0.85487108	12.6743036	1.48518762	0.09919977	0.01257752	0.42085351	0.03281627	3.00357369	0.01887099	6.45500000	0.00707107
M5	F	48	2	3.44928477	0.22401071	1.82136868	0.15163926	0.66494147	0.03757907	6.3946645	0.28011247	53.9150441	1.14138827	28.4579962	1.12476373	10.4212484	1.04415617	0.07205711	0.01221996	0.45906958	0.05795844	1.45166801	0.45083392	6.46000000	0.09899495
M5	F	72	5	5.19137463	0.51309039	2.82814636	0.30578574	1.14951548	0.13202661	9.8042581	0.90638251	52.9502909	1.80206903	28.8508864	1.77016565	11.7418304	1.09633750	0.06456992	0.00374646	0.63522167	0.09056333	2.34621923	0.83727494	6.38800000	0.01788854
M5	W	0	2	0.55589158	0.06911694	0.22211339	0.00342718	0.07208888	0.00975918	0.9843979	0.08100378	56.3721860	2.38250582	22.6543987	2.21232683	7.3070942	0.39010260	0.13666321	0.00560282	0.13430406	0.00555484	1.22201555	0.49899243	6.37500000	0.03535534
M5	W	4	2	1.42907298	0.16599435	0.43261626	0.01206846	0.18066122	0.03930700	2.0988507	0.17174554	67.9924098	2.34511288	20.6576898	1.11538204	8.5596598	1.17236393	0.02790241	0.02402095	0.05650024	0.04562427	2.03952488	0.12485349	6.40000000	0.07071068
M5	W	10	2	2.48221043	0.17999444	0.99499041	0.05233946	0.38218411	0.03287263	4.2446159	0.42929236	58.5641192	1.68252801	23.4990650	1.14357177	9.0108964	0.13689009	0.08925919	0.02962990	0.38523094	0.16408583	2.66977025	0.17212833	6.40500000	0.03535534
M5	W	24	2	3.26061670	0.12508050	1.59195408	0.07336161	0.78315876	0.06349590	6.1430129	0.01064504	53.0767764	1.94416738	25.9159481	1.23913743	12.7496875	1.05572159	0.08257588	0.00350692	0.50728336	0.02242206	3.35006983	0.12648904	6.42500000	0.06363961
M5	W	48	2	4.48101422	0.08572145	2.43459885	0.25653672	0.82343168	0.08728158	8.2596308	0.43602535	54.3002597	1.82867066	29.4349139	1.55204314	9.9553317	0.53118370	0.06309495	0.00254556	0.52058601	0.00648560	1.92898254	0.59750972	6.49000000	0.07071068
M5	W	72	5	5.42110104	0.17285170	3.00030548	0.10893129	1.15255028	0.04537099	10.1748874	0.22050329	53.2776637	1.17902253	29.4909732	1.02281093	11.3240803	0.20824380	0.05907283	0.00210557	0.60093065	0.02078309	2.38560990	0.34633968	6.40800000	0.03033150

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CHAPTER 4

The effect of fractionated psyllium (*Plantago ovata*) husk and whole seed flour on rice flour and starch cooking properties

Statement of Authorship

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Name of Principal Author (Candidate)	Tharuka Jayampathi		
Contribution to the Paper	Conceived the study, performed experiments, analysed, and interpreted data, wrote the manuscript		
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.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above).

ii. permission is granted for the candidate in 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.

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The effect of fractionated psyllium (*Plantago ovata*) husk and whole seed flour on rice flour and starch cooking properties

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Abstract (250 Words)

Wetted seeds of many plant species release viscous polysaccharide-rich gels called mucilage which are emerging as important food hydrocolloid ingredients. *Plantago ovata* seed mucilage has excellent properties as a food hydrocolloid but narrow plant genetic variation means that variability in technofunctionality is limited and innovative strategies for producing additives with tailored outcomes are needed. Here, we produced three fractions, namely cold water soluble, hot water soluble and residue, from *P. ovata* husk and whole seed flour (WSF) and assessed their influence on starch cooking using rapid-visco analysis (RVA) and gluten-free (GF) baking. The addition of husk, WSF or each derived fraction individually produced wide differences in RVA profiles of rice flour or rice starch combinations. Unfractionated husk and WSF reduced the pasting temperature and overall paste viscosity and produced high-quality GF bread. More soluble fractions contributed to starch paste retrogradation rather than enhancing hot paste viscosity but improved the texture and quality of GF bread. Comparatively, residue fractions significantly reduced pasting temperatures and increased paste viscosity in RVA tests and produced denser GF bread than controls. This was attributed to strong self-association within the fractions leading to phase separation from starch in the combined mixtures. Importantly, the function of each fraction was weaker than the corresponding whole unfractionated material suggesting that native assembly of the husk/WSF particles is necessary to provide maximum functionality in GF bread production. Thus, a balance between network formation, water binding and microstructural effects is likely to be key to effective GF bread production using *P. ovata.*

Introduction

Fractionation of polysaccharide mixtures obtained from various sources found in nature has the potential to provide polysaccharides with different structures, functionality and rheology useful as additives to tailor food product properties. Researchers have fractionated a number of such polysaccharide mixtures, producing a range of different products. Shi *et al.* (2017) fractionated polysaccharides from aloe vera leaf skin resulting in products with various structures and compositions and suggested their application as a low-cost gelling agent alternative in medicinal products and foods such as aloe jelly and drinks. Mandalari *et al.* (2005) produced arabinoxylan fractions from wheat bran and brewers' spent grain with different branching, solubilities and substitutions for food and non-food uses, according to their differing gelling behaviours. Wang *et al.* (2021) fractionated barley water-soluble fibre with different viscosities and gelling abilities and suggested the potential application of these fractions to tailor the properties of beverages and other liquid products and for use as thickeners and stabilizers.

Psyllium husk is used not only as a source of natural dietary fibre but is also widely employed in the pharmaceutical and food industries with major applications as a stabilizer in gluten-free bread (Ren, Linter, *et al.*, 2020). It is derived by milling the outer polysaccharide coating off commercially grown *Plantago ovata* seeds. Unmilled *P. ovata* seeds display at least three mucilage layers (L1, L2 and L3) when imbibed in water, producing a viscoelastic shell surrounding the seeds that allows water retention (Yu *et al.*, 2017). Xylose and arabinose are found in abundance within the seed mucilage, based on observations made by Fischer *et al.* (2004) and Marlett and Fischer (2003) who first determined that *P. ovata* mucilage polysaccharides are formed of arabinose-xylose branches bonded to a xylose backbone structure that they termed arabinoxylans, although this typically better describes cereal xylans. More accurately, *Plantago* mucilage polysaccharides are classed as heteroxylans where rhamnose and galacturonic acid are also found within the mucilage, as part of the substitutions, as outlined in the studies by Yu *et al.* (2017) and Cowley *et al.* (2020). Mannose, glucose and galactose are present in

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both the mucilage and seed tissues and are classified as fermentable sugars. Yu *et al*. (2017) performed fractionation based on mucilage density combined with temperature and alkaline extraction, producing the three sequential fractions: cold water (CW), hot water (HW) and alkaline soluble (KOH). The CW fraction, which is found in layer L1, is highly water soluble due partly to being rich in galacturonic acid residues. It was found that the average molecular weights of the polysaccharides were quite similar in all three fractions, however their hydrodynamic shapes seem to differ which was attributed to heavier branching, a smaller molecular volume through self-association and the presence of compact conformations in alkaline solutions. Although KOH fractions had a very similar composition to HW fractions and were both neutral, the HW fraction had a comparatively more open conformation and displayed differences in susceptibility to hydrolytic enzymes. Therefore, the distribution of side chains and atypical linkages in the main chain may give rise to the observed rheological differences between the HW and KOH fractions. Ren *et al.* (2020) performed temperature-dependent fractionation to reveal structural trends impacting solubilisation temperatures, extracting polymers from *P. ovata* seed husk (psyllium husk) at 20, 40, 60, 80, 100 °C. Of the temperature-separated fractions obtained, F20 had the highest yield after the residue was fractionated further into F40, F60, F80, F100 and dust (heat insoluble), where L1 = F20, L2 = F40 + F60, L3 = F80 + F100 + dust. Ren *et al.* (2020) noted that this cold soluble fraction (F20) had the lowest ratio of arabinose to xylose (A:X) at 0.298. In general, this would mean that for every xylose comprising the backbone, there are 0.298 arabinose side chain additions but for *P. ovata* this is complicated by the presence of xylose side chain additions, so A:X can only be used as a proxy or estimate for branching level. It was concluded that higher temperature fractions had greater A:X values and were thus more heavily substituted. It was observed that higher temperature-soluble fractions exerted more hydrogen bonding between side chains. The gelation mechanism of psyllium is very distinct from other polysaccharides and is mainly caused by hydrogen bonding and 'interlacing' between side chains rather than backbone-backbone junctions which are prevented from forming due to very high decoration of the backbone by side chains (Yu *et al.*, 2019). The distinct differences in the gel strength, rheology and melting properties of these comb-like

heteroxylan fractions could be caused by differences in hydrogen bonding capacity of xylopyranose and the arabinofuranose units, variation in carbohydrate sequences, xylose/arabinose arrangement of side chains and variation in substitution motifs (Yu *et al.*, 2018). Guo *et al.* (2008) divided psyllium mucilage into three fractions, namely a water extractable fraction WE, one alkali extracted fraction AES 0.5 which was also water soluble and AEG 0.5 which was water insoluble and constituted the majority of the mucilage. All fractions consisted primarily of xylose and arabinose but with key structural differences. WE and AEG 0.5 displayed 1→4 and 1→3 linkages in the main chain and the side chains connected by O-3 and O-2 linkages, while AES 0.5 had 1→3 linkages and side chains in the O-3 position. WE was found to be more branched with more complicated side chains while AES 0.5 was the simplest structurally. The three fractions displayed different gelling capacities. Fraction AEG formed a weak gel when in contact with water, and while its rheological properties were not compared with other fractions in the study, was deemed responsible for the gelling properties of psyllium husk. In addition, this gel fraction was found to have no sharp melting point or display thermal hysteresis. Guo *et al.* (2009) and Yu *et al.* (2017) made similar observations, showing that the CW fraction (comparable to Guo's WE fraction) had liquid-like behaviour (tan δ > 1) while the KOH fraction (comparable to Guo's AEG fraction) exhibited strong solid/gel-like behaviour (tan δ < 1). They also suggested that psyllium forms a 'physical gel' through sidechain interlacing rather than a chemical one where backbone junction zones are formed.

Cowley *et al.* (2020) sequentially extracted fractions from *P. ovata* seed using cold water, hot water and physical force and used microscopy to demonstrate how the mucilage layers were removed from around the seed at each extraction step. Based on observations from the fractionation and microscopy work, it is hypothesized that the *Plantago* mucilage forms a 3D network which reinforces the layered structure of the mucilage and imbues it with at least some of the properties which are advantageous to mucilaginous seeds (Cowley and Burton, 2021). The water affinity of seed mucilage aids in maintaining hydration during germination as well as modulating seed dispersal (Kreitschitz, Kovalev and Gorb, 2015; Pan, Girvin and LoPresti, 2022) and influencing the interactions between the seed and soil (Yang *et al.*, 2012;

Teixeira *et al.*, 2019; Pan *et al.*, 2021). Strong mucilage envelopes around seeds have been shown to greatly increase the chances of seedlings establishing in harsh environmental conditions (Witztum, 1978; Gutterman & Shem-Tov, 1996; Teixeira *et al.*, 2019), particularly those in arid areas and influenced by unstable weather patterns (Huang *et al.*, 2008; Cowley and Burton, 2021). Adaptive differences have been found among *Plantago* populations where increased stresses related to water deficiency led to higher seed mucilage contents (Teixeira *et al.*, 2019). Yu *et al.* (2017) suggests that the cold water soluble arabinoxylan fraction of psyllium drives an eruption of mucilage from the seed upon contact with water and undergoes gelation to form a viscoelastic shell which leads to retention of water molecules in close proximity to the seed itself. This model was corroborated by Phan *et al.* (2020). Therefore, the differences between the structure of the arabinoxylans in the different layers leads to formation of mucilage gel layers which may act as a water reservoir to provide a protective effect under environmental stresses such as heat, drought and salinity. However, although it has been hypothesized that the orchestration of the different mucilage layers is important for biological function in a plant growth context, it is unknown how important the layer organization is to the functionality and behaviour of *Plantago* mucilage in food systems. Therefore, in this study *P. ovata* husk, and for the first time, whole seed flour, were temperature fractionated, and the technofunctional influence of their addition to rice flour and starch was studied using rapid visco analysis (RVA) and baking experiments.

Materials and Methods

Materials

Plantago ovata whole seed flour (WSF) was produced and milled as per Cowley, O'Donovan and Burton (2021). Psyllium husk powder (VITACEL®) was obtained from JRS (J.Rettenmaier & Söhne Group, Rosenberg, Germany). Food grade psyllium was purchased from BuyWholeFoodsOnline.co.uk, originating in India. Caster sugar, salt and sunflower oil were purchased from Sainsbury's, UK. DCL instant dried yeast and Gluten-free Rice Flour were both donated by Elizabeth Starr, University of Nottingham. Rice flour was purchased from Doves Farms (United Kingdom). Rice starch (S7260) for rapid visco analysis (RVA) was purchased from Sigma-Aldrich (Germany).

Fractionation of Psyllium WSF and husk

A 0.5 g amount of WSF or husk was added to 100 ml RO water in a 300 ml glass beaker and placed in a water bath. Using overhead stirrers rotating at 300 rpm, the material was mixed at 20 °C for 1 hour in order to solubilise all available cold-soluble fractions. Solutions were divided amongst 50 ml centrifugation bottles and spun for 20 minutes at 2999 RCF at 20 °C, using a Rotina 380R centrifuge.

After centrifugation, the supernatant (F1 -cold soluble) and pellet were separated. The pellet was redispersed in RO water and the extraction was repeated with a mixing temperature 60 °C whilst all other parameters remained the same. The supernatant was decanted (F2– heat soluble), and pellet (F3– heat insoluble) was separated. All fractions were decanted into aluminium trays and placed in the drying oven at 105 °C for a minimum of 24 hours (Table 4-1).

Each sample was weighed prior to and post-drying to calculate dry weight and percentage yields of cold soluble, heat soluble and heat insoluble fractions.

Fractionation was scaled up to extract sufficient weights of each fraction for RVA, monosaccharide analysis and bread making.

Monosaccharide analysis

Monosaccharide analysis of WSF and husk fractions was carried out using reversed-phase HPLC of 1-phenyl-3-methyl-5-pyrazoline (PMP) derivatives according to Comino *et al.* (2013) on a 1260LC (Agilent, USA) with modifications as follows. Solutions at 10% acetonitrile in 40mM ammonium acetate (pH 6.8) (A) and (B) 70% Acetonitrile were used as eluents with a total run time of 11.5 minutes at a constant flow rate of 0.8 ml/min in the following gradients: 92% A and 8% B for 9.3 minutes, 83% A and 17% B for 0.7 minutes, 100% B for 1.5 minutes.

Preparation of rice flour/*Plantago* flour suspensions

Twenty-five milligrams (1% addition) of husk and fractions or 100 mg (4% addition) of WSF was blended with 2.5 g of rice flour or rice starch in an RVA canister. Twenty-four grams of deionised water was added to the mixture and homogenised briefly by vigorous stirring. Control samples lacking *P. ovata* WSF, husk or fractions were prepared using the same protocol.

Measurement of pasting properties

Pasting properties of rice flour-WSF/husk/fractions and rice starch- blends WSF/husk/fractions were determined by Rapid Visco-Analysis using an RVA Super 4 (Newport Scientific, Australia) for standard temperature pasting (<100 °C) based on Diaz-Calderon *et al.* (2018) with modifications similar to Cowley (2020). Suspensions were prepared as above in the RVA canister, held at 25°C for 2 min before heating to 95 °C (standard temperature) 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-minute period. During the first minute, the suspension was homogenised at 960 RPM, followed by constant stirring at 160 RPM for the rest of the test.

The following pasting parameters were extracted in triplicate: pasting temperature, peak viscosity, trough viscosity, breakdown viscosity, final viscosity, and setback viscosity.

Preliminary baking performance of *Plantago ovata* husk and flour fractions

The impact of the addition of *P. ovata* husk, WSF, and fractions on the baking quality of gluten-free breads was assessed following a procedure and formulations based on Ren et al. (2020) (Table 4-2). Water and oil were added to the dry ingredients and mixed in a multi-speed tilt-head stand mixer (Chef Premier, Kenwood Ltd, UK) with a silicone-edged creaming beater (AT501, Kenwood Ltd) for 7 minutes at a constant speed. With exact mass recorded, 230–240 g of dough was transferred to a medium-sized disposable aluminium tray lined with sunflower oil and rice starch. Loaves were proofed for 85 minutes at 30 °C before baking at 230 °C for 40 minutes in a deck oven (Compacta, Tom Chandley, UK). Loaves were cooled for 1 hr before measurements were taken.

Loaves were cut into 20 mm-thick slices and a mid-slice loaf height and median crumb brightness were measured from a representative central slice using a C-Cell Bread Imaging System (Calibre Control International Ltd., UK). Texture profile analysis (TPA) was performed on a representative slice using a texture analyser (TA.HD plus, Stable Micro Systems, UK) fitted with a 3mm indentation sphere. The hardness and springiness of the crumb was extracted using the standard TPA macro. Crumb moisture content was determined from a sample of crumb after drying at 105 °C for 24 hours.

Statistical Analysis

Statistical differences in pasting properties between samples and the control and fractions and their unfractionated counterparts were determined by independent Student's *t*-test in Microsoft Excel.

Results / Discussion

Physical properties of *P. ovata* husk and WSF fractions

The fractionation of *P. ovata* husk and whole seed flour (WSF) was carried out to yield three fractions F1, F2 and F3. The physical appearance of these dried fractions is shown in Supplementary Figure 4-1. The starting material of psyllium husk was off-white to light brown in colour. When stirred at 20 °C and then centrifuged the resulting supernatant was slightly viscous, free of particles and completely clear with a slight yellowish hue. The HF1 (F1 from husk) fraction dried to form a pale yellowish-brown glossy film that could easily be peeled from the container it was dried in. The WF2 (F2 from WSF) fraction, originally extracted at 60 °C, was very viscous with a jelly-like consistency, transparent, particle free and almost colourless. Once it was dried it formed a film which was glossy but lighter in colour than HF1. HF2 (F2 from husk) had the lowest yield out of the three fractions. The pellet remaining after extracting HF3 (F3 from husk) was dense and gel-like containing large amounts of suspended insoluble particles giving it a brown colour. Once dried this was dull brown with a rough texture. HF3 was the largest of the three fractions. Of all the dried materials HF1 was darker in colour since it was extracted first and therefore contained a larger amount of pigments that changed colour during high-temperature drying (Supplementary Figure 4-1). Cowley *et al.* (2022) states that non-husk tissues of *P. ovata* seeds contain colour-changing pigments, which may affect organoleptic properties when applied in food systems. The darker colour of HF2 was due to pigments extracted from low levels of contaminant non-husk tissues in the husk sample while that of HF3 was primarily due to presence of the insoluble particles.

The fractionation of *P. ovata* WSF was carried out as for husk fractionation. The WF1 separated from the supernatant was much less viscous compared to the HF1 fraction but clear and free of particles. It also appeared browner in colour while in solution. During the drying process the colour changed to black so the final material was glossy dark black and film-like. HF2 when in solution was more viscous than HF1 but less viscous compared to the jelly-like consistency of HF3 (Supplementary Figure 4-1). It dried

into a light brown glossy film. The WF1 fraction was much more pigmented as it was extracted first and thus contained more concentrated pigments. The subsequent fractions did not contain so much pigment. WF3 was the largest of the fractions, similar to HF3. However, it was darker and had a sandy, rough texture. It didn't contain insoluble particles suspended in a thick gel as for HF3, as it had a less gel-like property with more grainy insoluble material. WF3 appeared to have the least polysaccharide material and highest insoluble particulate material out of all fractions. Therefore, when applied in similar quantities to the other fractions to pasting of rice flour and rice starch experiments, they may show different properties which will be discussed in the context of rapid visco analysis.

Monosaccharide composition of *Plantago ovata* WSF and husk fractions

Monosaccharide analysis of the fractionated husk and whole seed flour of *P. ovata* revealed that xylose and arabinose are the major components of each mucilage fraction as shown in Table 4-3, similar to previous findings (Cowley *et al.*, 2020; Phan *et al.*, 2016; Yu *et al.*, 2017; Guo *et al.*, 2008). Guo *et al.* (2008) suggests that there is a variable monosaccharide composition between the fractions which gives rise to different structural features, although the abundance of xylose and arabinose in all fractions suggest a similar main structure of heteroxylan, where a xylose backbone is heavily substituted by arabinose. Significant amounts of rhamnose and galacturonic acid were found in the fractions extracted first in cold water (HF1 and WF1) of both husk and WSF, which indicates the presence of pectin (Table 4-1) similar to reports by Cowley *et al.* (2020) and Phan *et al.* (2016). Both second fractions (HF2 and WF2) had lower amounts of galacturonic acid and none was present in HF3 or WF3. Guo *et al.* (2008) made similar observations regarding uronic acid content in WE, which is water soluble and the AES0.5 fraction which dissolves in 0.5 M NaOH, but none was present in AEG0.5 which is insoluble in water and 0.5 M NaOH. Interestingly the A:X ratio varied significantly, rising with the extraction intensity required to yield the husk fractions, with the lowest A:X for the highly soluble HF1 extracted in cold water and the highest for HF3 which is a residual fraction with low solubility in hot or cold water. The same trend was observed for WSF fractions although A:X was higher in WSF fractions overall

compared to corresponding husk fractions. Yu *et al.* (2017) observed increasing A:X in cold water, hot water, and KOH fractions whilst Ren *et al.* (2020) reported increasing A:X in higher temperature-extracted fractions. Many arabinoxylans from cereals with simple structures have increased solubility with a higher A:X, since side chains containing arabinose interfere with the packing of xylan backbones (Mandalari *et al.*, 2005). By contrast, Ren *et al.* (2020) suggests that psyllium mucilage fractions are less extractable with increasing A:X and suggests the solubility of psyllium is dependent on factors other than changes in backbone packing. A:X increases correlated with rising resistance to extraction were also observed by Guo *et al.* (2008), Yu *et al.* (2017) and Cowley *et al.* (2020). The higher A:X of WSF fractions in comparison (Table 4-1) to its husk fraction counterpart may suggest that the WSF fractions are less soluble in comparison to each respective husk fraction. The increase of A:X indicates more complex branching in the difficult-to-extract fractions and also those fractions likely have stronger water-binding properties. Due to the variation in A:X across fractions, average A:X in the sum of fractions is still lower than the HF3 and WF3 fractions. These differences may impart different functionalities of the fractions and the way that they affect starch cooking properties.

Pasting properties of rice starch and rice flour blended with *Plantago* WSF and husk fractions

Pasting Temperature

Heating of rice flour-water suspensions leads to pasting, embodied by a sudden increase in viscosity, and mimics the cooking process in starch-based foods. The starch granules absorb water, swell and finally rupture, leaching out amylose to produce a viscous paste (Sandhu, Singh and Malhi, 2007; Batey, 2007) which has a continuous phase of dissolved starch molecules and a dispersed phase comprising granule fragments and swollen intact starch granules (Bemiller, 2011). The temperature at which the viscosity increases is the pasting temperature and precedes the gelatinization temperature; the viscosity usually increases after the starch granules are completely gelatinized. Peak time is when the starch paste reaches its peak viscosity (Zhou *et al.*, 2007) and peak temperature is when the starch granules reach maximum swelling (Batey 2007; Balet *et al.*, 2019). In the absence of hydrocolloids, the

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swelling process is controlled by the starch micellar networks, orchestrated by the interaction of amylose and amylopectin molecules (Rosell, Yokoyama and Shoemaker, 2011; Rojas, Rosell and Barber, 1999). During gelatinization, a continuous phase is formed by the leached amylose and low molecular weight amylopectin which is dispersed amongst swollen starch granules. Alternatively, in the presence of hydrocolloids a phase separation is seen where a hydrocolloid-rich continuous phase is isolated within amylose and amylopectin rich domains (Alloncle *et al.*, 1989). Sikora, Kowalski and Tomasik (2008) concluded that gelation of starch in composite pastes proceeds under conditions of water deficiency, particularly when the starch/hydrocolloids are present in higher concentrations and/or lower water is found in the medium. During gelatinization the leached amylose and low molecular weight amylopectin interact with the hydrocolloids present in the continuous phase resulting in the alteration of pasting depending on the type and concentration of hydrocolloid (Shi and BeMiller, 2002). These interactions can be synergistic and result in composites with superior properties to the individual components alone, which is advantageous in food formulation scenarios. There is limited literature available on the effect of psyllium addition to the pasting properties of rice flour (Cappa, Lucisano and Mariotti, 2013; Mancebo *et al.*, 2015; Cowley, 2020). Here, the addition of unfractionated husk or HF3 caused a significant reduction of pasting temperature in comparison to the control (p<0.05) (Figure 4-1). Cowley (2020) also observed a reduction of pasting temperatures of rice starch and rice flour upon addition of unfractionated *Plantago* WSF. HF1 and HF2-augmented mixtures however did not show a significant difference from control (p>0.05). The performance of fractions HF1 and HF2 were significantly different from the unfractionated husk, while HF3 was similar to husk. Of the three fractions, HF3 displayed the largest degree of pasting temperature reduction, possibly due to its higher water binding ability as related to its higher A:X (Table 4-1). WF1 and WF2 exhibited different attributes compared to unfractionated WSF (P<0.05) as shown in Figure 4-2). WF3 showed a similar reduction of pasting temperature to WSF, which was much higher than WF2 and WF1. Like WF2, HF2 did not produce a significant difference from the control (p>0.05). In rice starch pastes the presence of HF1, HF3 or whole husk all changed the pasting temperature significantly from the control (p<0.05) and to the same extent

(Figure 4-3). WSF fractions performed differently to WSF (P<0.05) as shown in Figure 4-4. The highest reduction of the pasting temperature was observed in WSF-rice starch blends where the addition of 4% WSF conferred the greatest reduction of pasting temperature at >5°C, probably due to the less complex nature of rice starch in comparison to rice flour, as shown in Figure 4-4. However, F1 and F2 fractions provided weaker reductions in pasting temperatures (Figure 4-5 and 4-6) in comparison to unfractionated husk, WSF, HF3 and WF3, which suggests that the polysaccharides responsible for the reduction of pasting temperature in the WSF and husk are concentrated in the F3 fraction.

Mancebo *et al.* (2015) also noticed a reduction of pasting temperatures at lower addition rates of psyllium into rice flour pastes. Similar observations were made by Ren, Linter and Foster, (2021) who suggests that this may be due to water and volume competition between starch and psyllium hydrocolloids. The presence of xanthan gum has been shown to enhance the initial stages of granule swelling due to higher viscosity in the suspension (Christianson *et al.*, 1981; Sullo and Foster, 2010). Similarly, the high water affinity of psyllium polysaccharides may increase the viscosity of the continuous phase of dispersed hydrocolloids, hastening the initial stages of starch granule swelling. Díaz-Calderón *et al.*, (2018) concludes that small quantities of amylose leached during early stages of starch granule swelling could interact with hydrocolloids thereby enhancing the pasting. Such an interaction is hypothesised to occur in mixtures of native corn starch and xanthan gum hydrocolloids Zhang *et al.* (2018). Polysaccharides from some of the psyllium mucilage fractions seem to show a similar interaction with leached amylose thereby hastening the pasting. Cowley *et al*. (2020) hypothesised that the hastening of pasting onset upon WSF addition from different *Plantago* species was at least partly a result of amylose interactions with heteroxylan. Such interactions in the presence of such high levels of backbone substitution (which typically prohibit strong heterotypic associations) were hypothesised to be a result of helical associations from regularly substituted domains which allow conformations where backbone is available for interaction in the presence of high substitution.

Peak Viscosity

Addition of WSF and husk had a large impact on the peak viscosity of rice flour with a >30% increase compared to the control (Figure 4-1 and 4-2). HF1, HF2 and HF3 performed significantly differently to unfractionated husk added to a rice flour paste (P<0.05) as shown in Figure 4-1 but HF1 and HF2 did not show a significant difference from the control (P>0.05). HF3 and unfractionated husk displayed the highest peak viscosity changes in rice flour paste. Similarly, WF1, WF2 and WF3 imparted significant differences in peak viscosity as compared to unfractionated WSF, shown in Figure 4-2. While WF1 and WF2 showed no significant difference from the control, WF3 rice flour pastes showed the highest peak viscosity out of the three WSF fractions. For husk fractions and rice starch pastes, whole husk and HF3 had a similar viscosity (P<0.05), as demonstrated in Figure 4-3. HF3 conferred a higher peak viscosity than HF1. Peak viscosity of rice starch pastes supplemented with WF1 and WF3 (Figure 4-4) were significantly different when WSF was added. Again, the addition of WF3 gave rise to a higher peak viscosity in rice starch pastes than WF1. The F1 and F2 fractions with lower branching and water affinity seem to have little or no effect on starch breakdown during the pasting stage. However, the F3 fraction with higher branching and containing abundant particulate matter could be self-associating, leading to a phase separation and water-reduced conditions in the starch phase, encouraging starch-starch homotypic interactions and thereby increasing the viscosity in the medium during the cooking stage.

For rice starch pastes, the inclusion of WSF, husk or HF3 prompted an increase in viscosity >10% (Figure 4-3 and 4-4), but when WF3 was added the viscosity increase was less. Fractions F1 and F2 did not cause any considerable increase in the viscosity of both rice starch and flour pastes. This suggests that F3 fractions possess stronger water binding abilities compared to F1 and F2 and that most of the water binding and viscosifying effects of WSF and husk is imparted by the polysaccharides found in the F3 fraction. F3 fractions have higher solubilisation temperatures and Ren *et al.*, (2020b) found these fractions to have higher A:X ratios with greater degrees of substitution, thus creating the

comb-like structure. The viscosity peak of samples containing F3 was sharper than F1 or F2 mixtures, suggesting an exponential increase of junction zones, possibly due to a different fold structure which would affect binary gel formation. F2 fractions produced the lowest final viscosity value, with a significant difference between them and their F3 counterparts. Ren *et al.* (2020b) proposed that F2 displays a twisted ribbon structure as opposed to the F3 fractions which undergo a coil to helix transformation. Given that F3 fractions comprise the majority of the WSF and HUSK materials, (70.22% and 64.66%, respectively) these results indicate that the behaviours in mixtures are driven mainly by the F3 fractions, indicating that they are mostly responsible for the viscosifying ability of psyllium.

Mancebo *et al.* (2015) observed a slight increase in peak viscosity with low level additions of unfractionated psyllium husk to rice flour. Similarly, Cappa, Lucisano and Mariotti, (2013) observed an increase in peak viscosity when psyllium was added to gluten free dough also containing sugar beet fibre. These increases are due to the strong hydrophilic nature of the polysaccharides in psyllium which impart a high-water binding ability (Cappa, Lucisano and Mariotti, 2013; Mancebo *et al.*, 2015). Alloncle and Doublier, (1991) described hydrocolloid-starch blends as a system where the starch granules are dispersed in a continuous phase of hydrocolloid solution. Therefore, peak viscosity could be affected by changes in each phase as well as the interactions between them. These sort of systems containing strong water-binding hydrocolloids could thicken the starch suspension and increase the forces affecting starch granules, thereby hastening the onset of pasting viscosity as well as increasing the final peak viscosity (Christianson *et al.*, 1981). Sullo and Foster, (2010) state that pasting properties of starch hydrocolloid blends not only depend on the viscosity and behaviour of each component but also to the interactions between the components. The whole husk, WSF, HF3 and WF3 display the largest increases in peak viscosity. The A/X ratios of these are higher than the F1 and F2 fractions (Table 4-1), which suggests they have stronger water binding properties. Therefore, the increased peak viscosity could be due to the increased effective concentration of starch granules due to the high-water affinity of psyllium mucilage fractions promoting starch-starch interactions as well as interactions between the starch granules and the mucilage polysaccharides.

Trough and Breakdown Viscosity

When starch pastes in the RVA are maintained at the highest temperature (holding period) the viscosity reduces due to thixotropy leading to a trough in viscosity. At the pasting temperature, the starch granules absorb a lot of water which then leads to melting of the crystalline regions of the starch granule, promoting rapid entry of water into the granule and rapid swelling before the rupture and disintegration of granules, reducing the volume occupied by the granules and thereby decreasing the viscosity during the holding period (Batey, 2007).

The breakdown viscosity is the difference between the peak viscosity and the trough viscosity which is the lowest viscosity reached during the holding stage. Addition of unfractionated husk and HF3 into rice flour pastes led to a significant increase in trough viscosity ($P<0.05$) while HF2 addition was similar to the control as shown in Figure 4-1. Trough viscosities resulting from adding WF1, WF2 and WF3 to rice flour were significantly different from that of the unfractionated WSF (Figure 4-2). WF1 and WF2 did not increase trough viscosity compared to the control, and although WF3 promoted some increase, it was less than the change caused by the addition of unfractionated WSF. HF3 and unfractionated husk caused a similar increase of trough viscosity in rice starch mixtures ($P>0.05$) as shown in Figure 4-3. WF1 and both 4% WSF and 1% husk-rice flour blends maintained a trough viscosity over 20% higher than the control, also much higher than the F1 and F2 fractions derived from both WSF and husk (Figure 4-1 and 4-2). WF3 and HF3 fractions promoted the higher viscosity compared to other fractions. WF3 and HF3 had a similar effect to their unfractionated counterparts on trough viscosity (Figure 4-1, 4-2 and 4-5). WF3 showed a significantly different effect on trough viscosity when compared to unfractionated WSF in rice starch pastes as shown in Figure 4-4. Here again, WF3 increased the trough viscosity but to a lesser degree compared to unfractionated WSF while HF3 showed a similar performance to unfractionated husk. The lower viscosifying ability of WF3 could be due to the higher content of insoluble particulate matter present in the fraction which proportionally lowers the percentage of polysaccharides. Husk, WSF and HF3 promote the highest resistance to disintegration of the starch

network. Similarly, husk, WSF and HF3 maintained a higher viscosity in rice starch mucilage blends, with HF3 responsible for significantly higher viscosity than the others (Figure 4-3, 4-4 and 4-6). F1 and F2 fractions had no significant effect on the trough viscosity of both rice flour and rice starch pastes.

Unfractionated husk at a 1% addition level displayed a higher breakdown viscosity than 4% WSF in both rice flour and rice starch (Figure 4-5 and 4-6). The breakdown viscosity of both WF3 and HF3 were much higher than the F1 and F2 fractions in rice flour pastes but lower than their whole mucilage counterparts (Figure 4-1 and 4-2). In rice starch pastes, HF1 and HF3 did not show a significant difference from husk (P>0.05) or from the control (Figure 4-3). WSF, WF1 and WF3 addition did not change the breakdown viscosity significantly from the control (Figure 4-4). Trough and breakdown viscosity can be affected by various factors such as shear force, composition of the starch paste, type of starch used, and temperature gradients (Schirmer, Jekle and Becker, 2015; Copeland *et al.*, 2009). Satrapai and Suphantharika, (2007) concluded that hydrocolloids reduce water availability for granule swelling and increase shear force exerted on swollen granules, increasing breakdown.

Final and Setback Viscosity

Setback viscosity is determined by the difference between the peak viscosity and the final viscosity (Zhou *et al.*, 2007). A cooling stage follows the holding period of the RVA during which the starch granules cool down leading to an increase in viscosity and retrogradation then occurs (Batey, 2007). During this stage the starch paste forms a more crystalline structure due to the realignment of amylose and amylopectin. Kaur *et al.* (2007) states that during the cooling period, the viscosity actually increases, indicating the tendency of amylose in the hot starch paste to reassociate, driven by the reduction in the temperature and entropy. Sandhu, Singh and Malhi, (2007) suggests that a lower tendency of starch retrogradation is indicated by lower setback viscosities. The addition of 4% WSF and 1% husk to rice flour increased the final viscosity by about 20% from the rice flour control (Figure 4-2). HF1, HF2, HF3, WF1, WF2 and WF3 fractions led to an increase in the final viscosity in comparison to the control. However, husk fractions gave a much higher increase in final viscosity compared to the

WSF fractions. In contrast, in rice starch blends, addition of fractions did not significantly change the final viscosity (Figure 4-4).

Addition of fractions increased the setback viscosity of all rice flour and some rice starch mucilage blends (husk, HF1 and WF1) (Figure 4-1, 4-2, 4-3 and 4-4). However, addition of HF1 and HF2 led to a higher setback than unfractionated husk and HF3 in rice flour. WF1 and WF2 also increased the setback viscosity but to a lower degree than WSF. In rice starch pastes HF1 gave rise to higher setbacks than whole mucilage and other fractions. Although the F1 and F2 fractions had a lesser impact compared to F3 throughout the cooking stage, of all the starch-mucilage blend combinations they seem to have had a greater impact on the gelling of the starch matrix after cooking and the viscoelastic properties of the cooked starch, compared to F3. Cappa, Lucisano and Mariotti, (2013) also observed an increase in the setback viscosity with the addition of psyllium husk. It has been reported that increased concentrations of complex polysaccharides interacting with amylose leached during the starch pasting (Yuris *et al.*, 2017) and the interaction between amyloses as well as between amylose and hydrocolloids, can lead to increased gelation of starch-hydrocolloid blends (Huang *et al.*, 2007). Ptaszek and Grzesik, (2007) suggests that temperature dependent phase separation occurs in starch hydrocolloid blends to increase the gelation. The increased final viscosities and setback viscosities observed in both rice flour and rice starch with the addition of WSF, husk, and fractions F1, F2 and F3 could therefore be due to synergistic effects of hydrocolloid-amylose networks, amylose-amylose networks as well as temperature dependent phase separation.

Atypical peak during breakdown in *P. ovata*

An atypical peak was observed (~85 °C) in blends of rice flour with WSF, husk, HF3 and WF3 (Figure 4-5). The peak was a similar magnitude where WSF and husk were added whilst HF3 and WF3 fractions caused a smaller peak. However, HF3 developed a much stronger atypical peak than WF3.This peak was not observed in rice starch blends with husk, WSF or any of the fractions (Figure 4-6). F1 and F2 fractions did not cause this peak with either rice flour or rice starch (Figure 4-5 and 4-6). There is limited

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literature available on the pasting properties of *P. ovata*-starch blends (Habilla *et al.*, 2011; Cappa, Lucisano and Mariotti, 2013; Mancebo *et al.*, 2015; Cowley, 2020) with even fewer presenting the RVA profiles. RVA profiles from (Cappa, Lucisano and Mariotti, 2013) displayed similar atypical peaks in psyllium rice starch blends, although its origin was not explained. A similar secondary peak was also observed in pasting profiles of rice flour psyllium blends at approximately 85 °C (Ren, Linter, *et al.*, 2020; Ren, Linter and Foster, 2021) who suggest that this peak maybe due to the formation of weak gel particles of psyllium or temporary interactions of them with amylose. They observed an increase in the intensity of this peak with addition of increased amounts of psyllium, suggesting a correlation. Cowley (2020) also observed a similar atypical peak in *P. ovata* WSF and rice flour blends which was not observed for WSF of other species of *Plantago*, suggesting a unique transitory complex forms between polysaccharides of *P. ovata* and amylose at this temperature. Observations from this study suggests that polysaccharides which are concentrated in the F3 fractions of husk or WSF are the mucilage component responsible for this atypical peak, as it was observed in husk, WSF and F3 fractions but not in the F1, and F2 fractions extracted at lower temperatures.

Baking performance

To complement data about how different *P. ovata* husk and WSF fractions influence starch cooking behaviour, we performed a preliminary assessment of their influence on the properties of rice flour-based gluten-free (GF) breads (Figure 4-7).

When made with no added hydrocolloids, the control GF bread batter/dough was too fluid to retain proofing gases and thus was unable to rise effectively, producing a flat loaf (Figure 4-7A) with a low mid-slice height (Figure 4-7B). To improve the quality of this GF bread formulation, *P. ovata* husk, WSF, and the three isolated fractions thereof, were added individually to the formulation at the 4% level. The addition of husk and WSF increased the mid-slice height of the GF breads, as did the F1 and F2 fractions from both WSF and husk (Figure 4-7B). The increase in mid-slice height corresponded with the trapping of CO_2 bubbles during proofing which produced a concomitant reduction in loaf hardness

(Figure 4-7D) and increase in loaf springiness (Figure 4-7E). It was noted that F3 derived from both husk and WSF had drastically different influences on bread properties compared to the other fractions. WF3 produced a loaf with similar mid-slice height to the control, while HF3 reduced mid-slice height by *c.* 15% compared to the control (Figure 4-7B). These denser loaves had increased hardness (Figure 4-7D; HF3, +68%; WF3, +4.1%) and reduced springiness (Figure 4-7E; HF3, -14%; WF3, -2.5%) compared to controls. When considering all formulations, a larger mid-slice height logically positively correlated with crumb moisture and springiness and was negatively correlated with crumb hardness (Figure 4-7F). It was also observed that the addition of husk, WSF, and their fractions lead to a crumb darkening (Figure 4-7G), with husk and its derived fractions causing relatively small changes, and WSF and its fractions causing more substantial and varied changes. Most notably, F1 WSF darkened the crumb by *c.* 74%, around 50% more than WSF, WF2 and WF3. As the fractionation was performed sequentially, the compound(s) causing the significant colour change were likely concentrated and extracted during F1 production leaving less to go into F2 and F3. Cowley *et al.* (2022) reported colour-changing pigments to be a significant hallmark of environmentally damaged seeds, and the leaching of said pigments from below the mucilage layer is considered a significantly problematic factor in starch-based foods due to pH-dependent colour changes. These pigments are soluble in ethanol (Cowley *et al.* 2022) and it is possible that they can be removed or isolated during fractionation processes to circumvent this issue if the properties of a specific fraction are desired.

In these supplemented GF breads, the polysaccharides in each fraction may interact synergistically. While several fractions (HF1, HF2, WF1 and WF2) produced acceptable GF loaves, no fraction was equal to the performance of the corresponding unfractionated material. There are few comparable studies in the literature (comparison of temperature fractionated polysaccharides with starting material) but one study comparing dietary fibre fractions from rice bran similarly found that the effect of each isolated fraction was not equal to the whole unfractionated material (Phimolsiripol, Mukprasirt and Schoenlechner, 2012). F1 and F2 fractions are rich in polysaccharides, isolated by centrifugation, and do not contain solid insoluble particles, while F3 fractions contain the remnant insoluble components

from the unfractionated material. We previously speculated that anchoring of mucilage polysaccharides to the seed surface, and the macromolecular assembly this provides, are important to *Plantago* mucilage functionality (Cowley and Burton, 2021), so perhaps the polysaccharides in F1 and F2 synergistically anchor to the insoluble particles found in F3 in the unfractionated state. F3 fractions also contain the most highly branched heteroxylans (Cowley *et al*. 2021; Yu *et al*. 2017; Ren *et al*. 2020) which may positively affect mucilage-seed attachment in the native state but too strongly self-associate when other fractions are removed, leading to poor performance of the F3 fractions. Given that the concentration of polysaccharide in F3 fractions is lower than other fractions due to the higher concentration of remaining insoluble material, this will also affect F3 functionality. However, despite amounts of insoluble material in HF3 being lower compared to WF3, its more severe impact on bread quality makes the self-association hypothesis more likely. We therefore hypothesise that the native, layered assembly of the mucilage particles (in both husk and WSF), including insoluble structures, is integral to their in-food functionality, particularly in breadmaking. The fractionation process likely disrupts this assembly, negatively influencing the properties of the mixture.

Conclusions and Future Directions

In this study, the varied influences of *P. ovata* husk and WSF, and their three temperature-isolated fractions, F1, F2 and F3, were studied to unravel the influence of mucilage heteroxylan structure on starch cooking properties. We show that the separation of mucilage polysaccharides based on temperature solubility leads to isolation of polysaccharide components with different functionalities from the native state. In pasting experiments, F1 and F2 fractions showed a weaker effect compared to F3 on peak temperature, peak viscosity, trough viscosity and breakdown viscosity. However, although the performance of HF3 and WF3 was lower than their unfractionated counterparts, they were still much better than their respective F1 and F2 fractions. Likewise, in preliminary breadmaking tests, no fraction produced breads with quality as high as their whole unfractionated counterpart. Therefore, it is hypothesised that the native assembly of psyllium mucilage particles is necessary to provide maximum functionality. F1 and F2 fractions likely do not phase separate from starch during cooking which leads to synergistic gel formation during cooling and thus superior breadmaking performance compared to F3 fractions, which might self-associate too strongly (Figure 4-8). F3 fractions are therefore thought to play a more dominant role in the viscosifying ability of psyllium, which is tempered in unfractionated form by inter-fraction interactions (Figure 4-8).

A theme here is that the data show the influence of each fraction derived from both husk and WSF is weaker than the effect of adding unfractionated husk or WSF. Future experiments could be aimed at more discrete temperature fractionation and purification (such as that performed by Ren *et al.* 2020) to further assess the influence of heteroxylan structure on starch-based food production. It would also be informative to recombine these fractions (and those used in this study) at native ratios to further understand whether the synergistic interactions seen here are a result of native macromolecular assembly, which could be more firmly linked to their resultant properties and effects. This could provide a capability to predict the usefulness of naturally derived hydrocolloids on food cooking quality more accurately. We also suggest studying the changes to the rheology of the bread dough due to the

addition of mucilage fractions and the effect of different hydrations on the batters for baking bread. Carrying out sensory evaluations of the baked bread would also help to establish the consumer acceptability of baked bread with various mucilage fractions.

The work presented here illustrates the varied effect of each fraction on gluten-free breadmaking and starch pasting as a useful step in providing a toolbox of materials for more selectively manipulating product quality than simply adding unfractionated material alone.

Figures

Table 4-1. Psyllium husk and WSF fractions

	WSF	HUSK
Cold Soluble	WF1	HF1
Heat Soluble	WF2	HF2
Heat Insoluble	WF3	HF3

Table 4-2. Ingredients for gluten-free bread making

Ingredient	Amount
Rice Flour	100 g
Caster Sugar	5 g
Salt	2 g
Yeast	1.5 g
Water	120 g
Sunflower Oil	5 g
Plantago flour fraction	0 g or 4 g (control or 4% addition,
Ingredient	Amount

Table 4-3. Monosaccharide analysis of fractions extracted from *P.ovata* seed husk and whole seed flour.

	D-Man	D-Rha	D-GalAc	Xyl	Ara	X +A	A/X
HF1	2.8±0.2	4.7±0.3	6.7±0.6	41.4±1.2	7.6±0.3	49±1.4	0.183
HF2	nd	2.4±0.2	4.6±0.1	50.2±2.8	10.5±0.6	60.7±3.4	0.210
HF3	nd	nd	nd	31.3±0.7	8.3±0.2	39.7±0.8	0.265
Total	0.9	2.4	3.7	41.0	8.8	49.8	0.215
WF1	nd	2.3±0.0	4.5±0.2	19.8±0.7	4.5±0.2	24.4±0.8	0.230
WF2	nd	nd	3.6±0.1	29.2±0.3	6.9±0.1	36.1±0.4	0.236
WF3	nd	nd	nd	5.6±0.3	1.7±0.1	7.3±0.3	0.294
Total	nd	0.8	2.7	18.2	4.4	22.0	0.241

Values are mean ± standard deviation in %mol/mol. nd= not detected
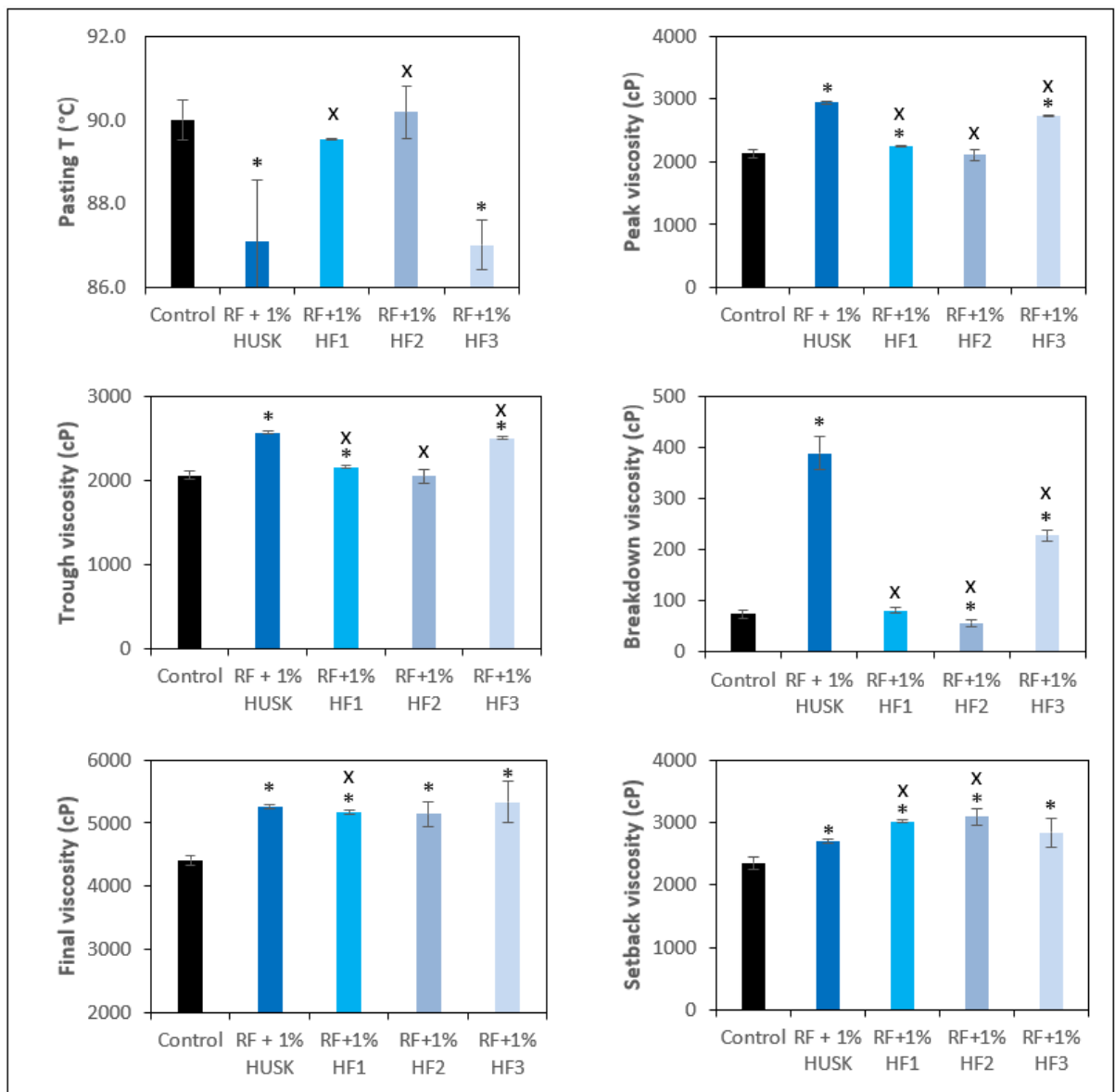
Figure 4-1. Pasting properties of rice flour (RF) upon addition of fractions extracted from *P. ovat*a husk. Values are means ± SD. Values for fractions that are significantly different from unfractionated sample are indicated by an x (p<0.05). Samples that are significantly different from control sample are indicated by an * (p<0.05).
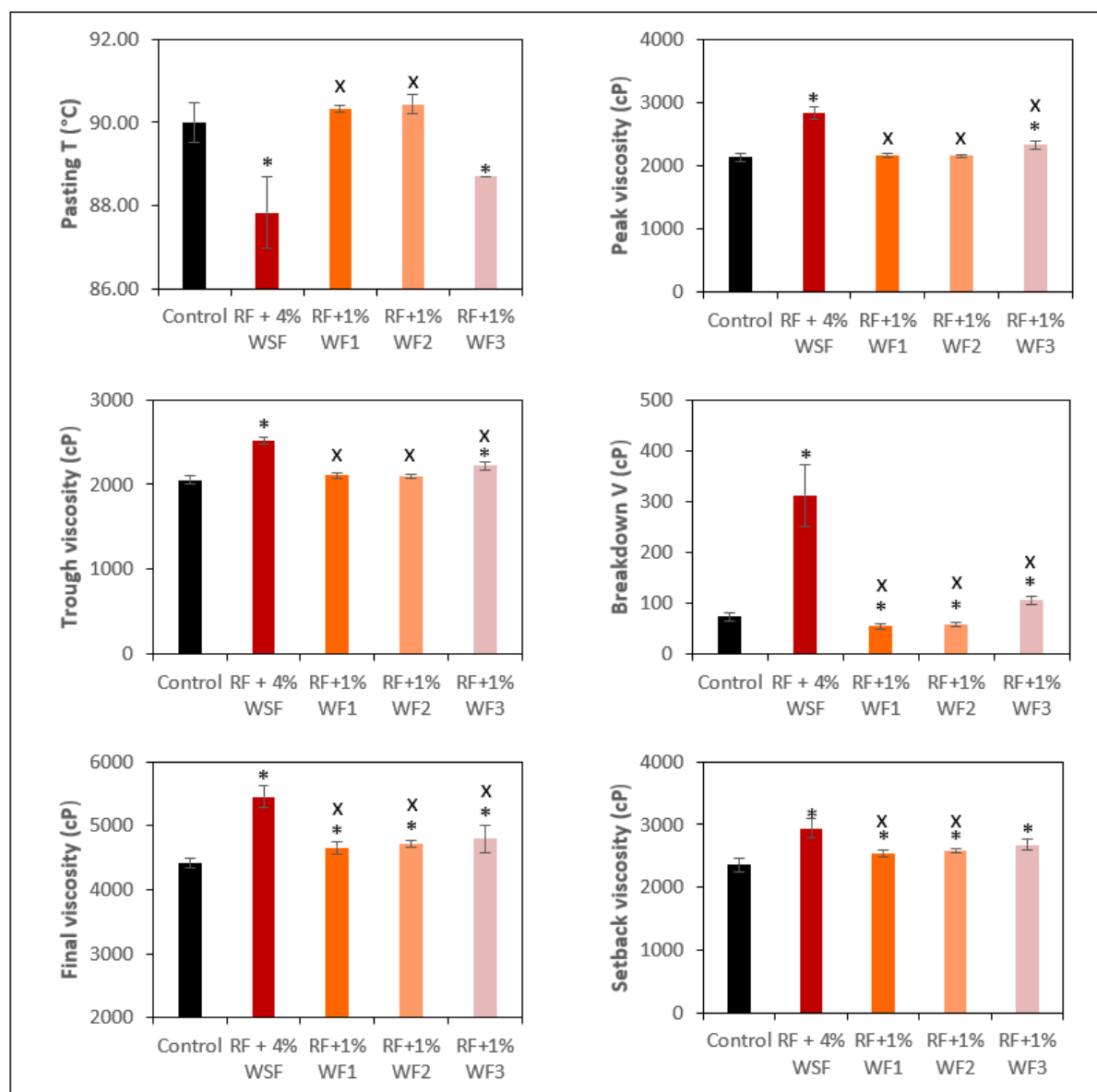
Figure 4-2. Pasting properties of rice flour (RF) upon addition of fractions extracted from *P. ovata* WSF. Values are means ± SD. Values for fractions that are significantly different from unfractionated sample are indicated by an x (p<0.05). Samples that are significantly different from control sample are indicated by an * (p<0.05).
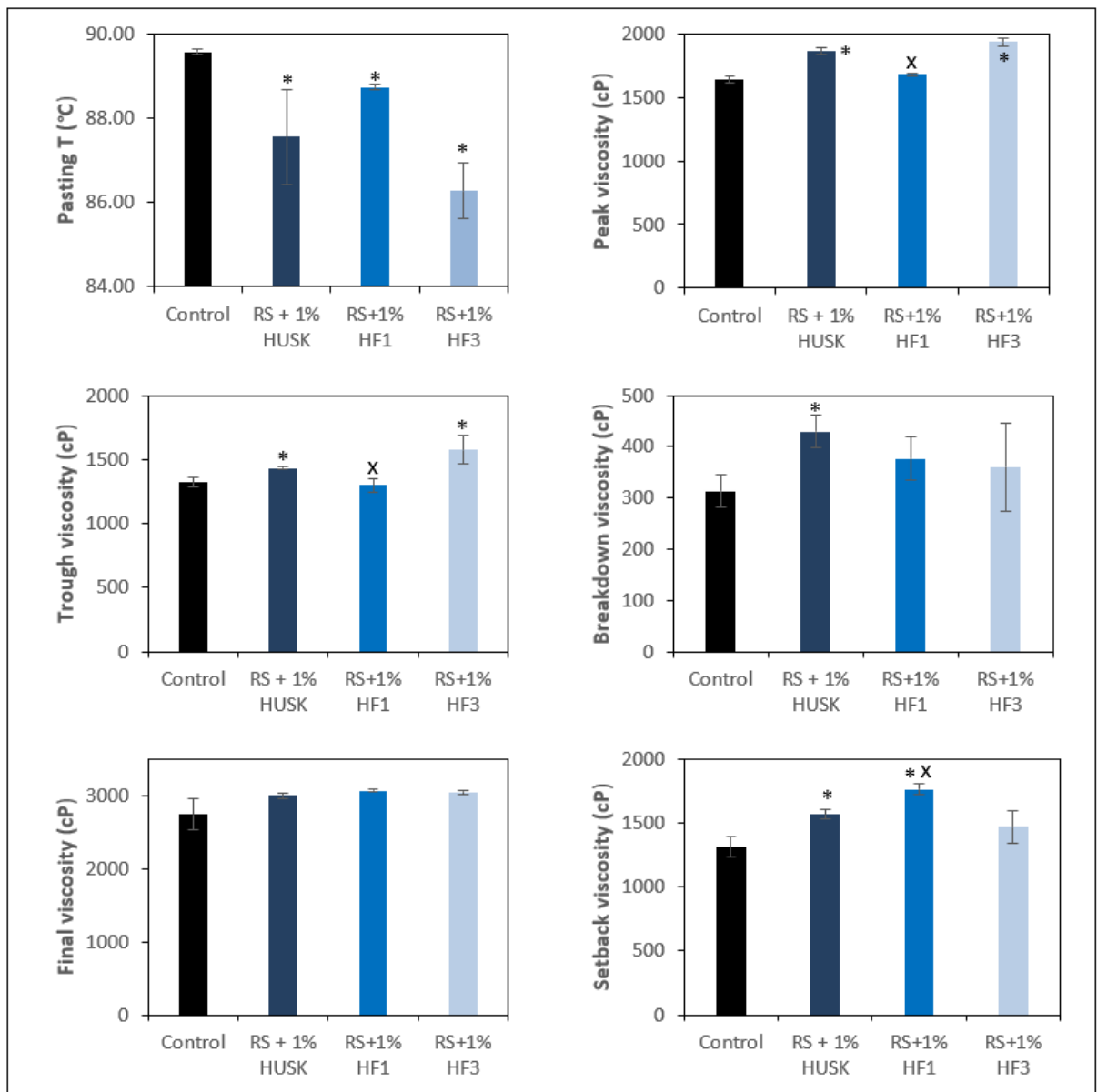
Figure 4-3. Pasting properties of rice starch (RS) upon addition of fractions extracted from *P. ovata* husk. Values are means ± SD. Values are means ± SD. HF2 data not available due to insufficient fraction sample. Values for fractions that are significantly different from unfractionated samples are indicated by an x ($p<0.05$). Samples that are significantly different from control samples are indicated by an * ($p<0.05$).
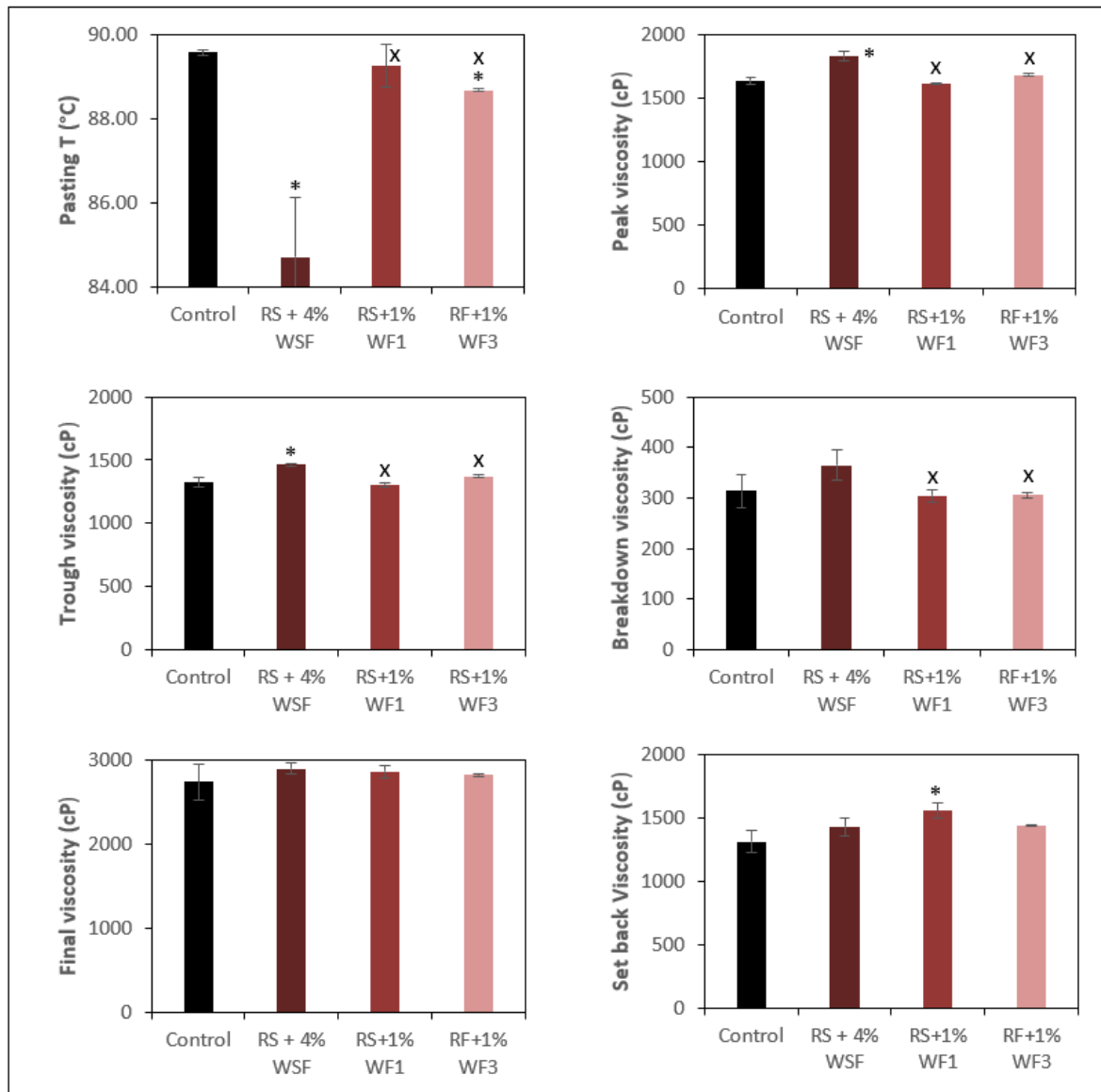
Figure 4-4. Pasting properties of rice starch (RS) upon addition of fractions extracted from *P. ovat*a WSF. WF2 data not available due to insufficient fraction sample. Values for fractions that are significantly different from unfractionated samples are indicated by an x (p<0.05). Samples that are significantly different from control samples are indicated by an * (p<0.05).
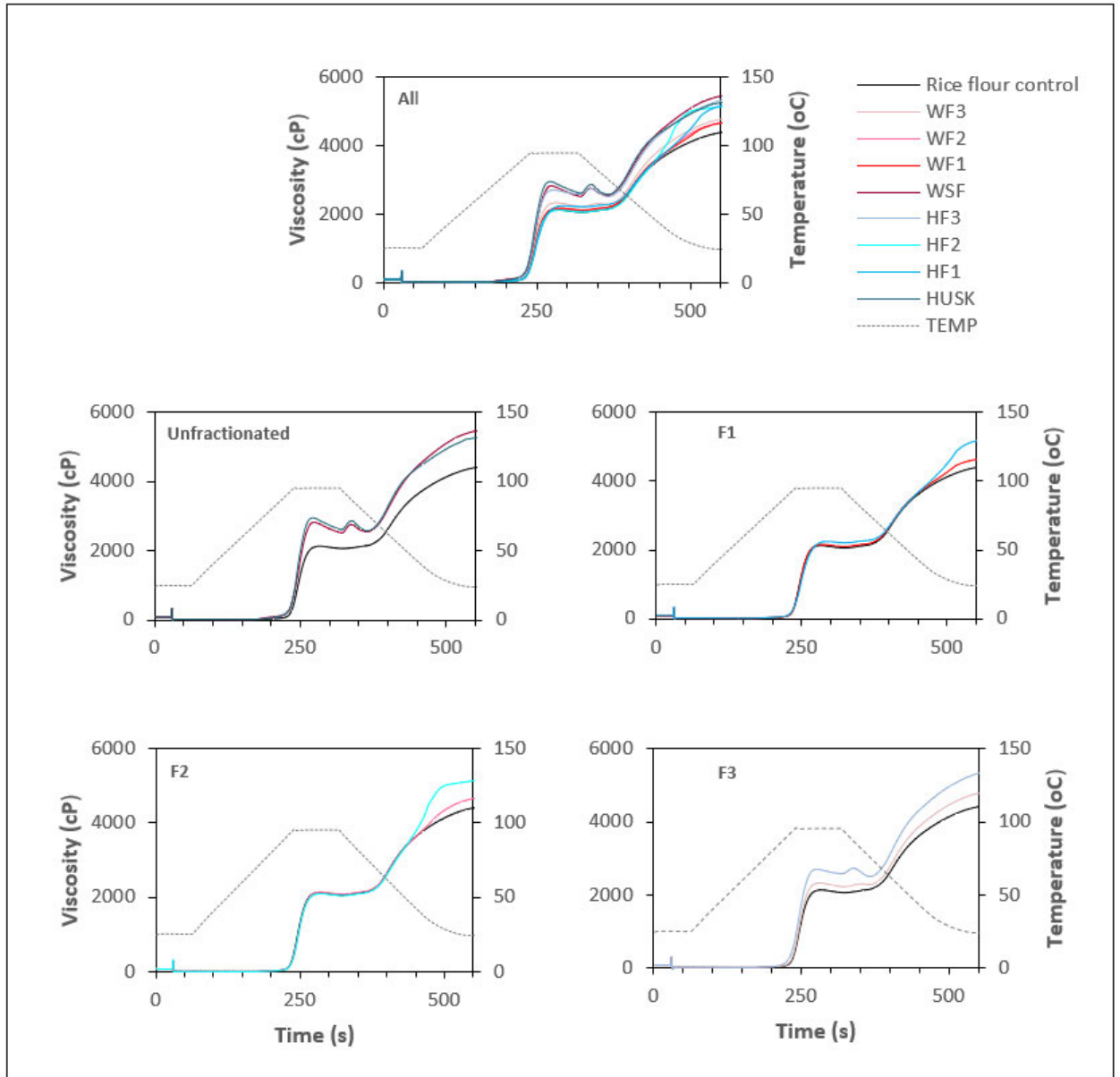
Figure 4-5. Change of RVA profile of rice flour with the addition of 1% *P. ovata* husk, 4% *P. ovata* WSF or 1% husk and WSF mucilage fractions
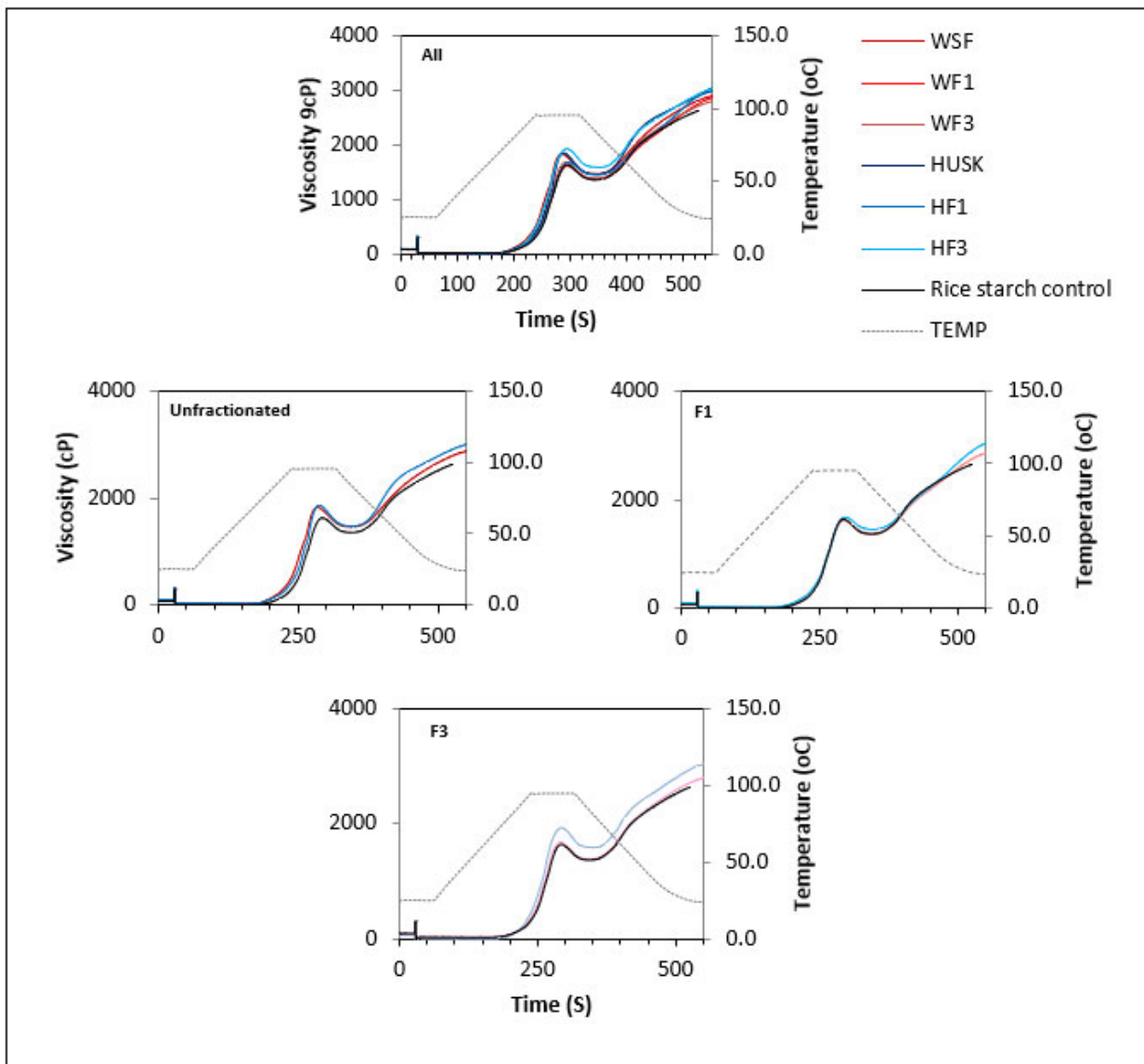
Figure 4-6. Change of RVA profile of rice starch with the addition of 1% *P. ovata* husk, 4% *P. ovata* WSF or 1% husk and WSF mucilage fractions
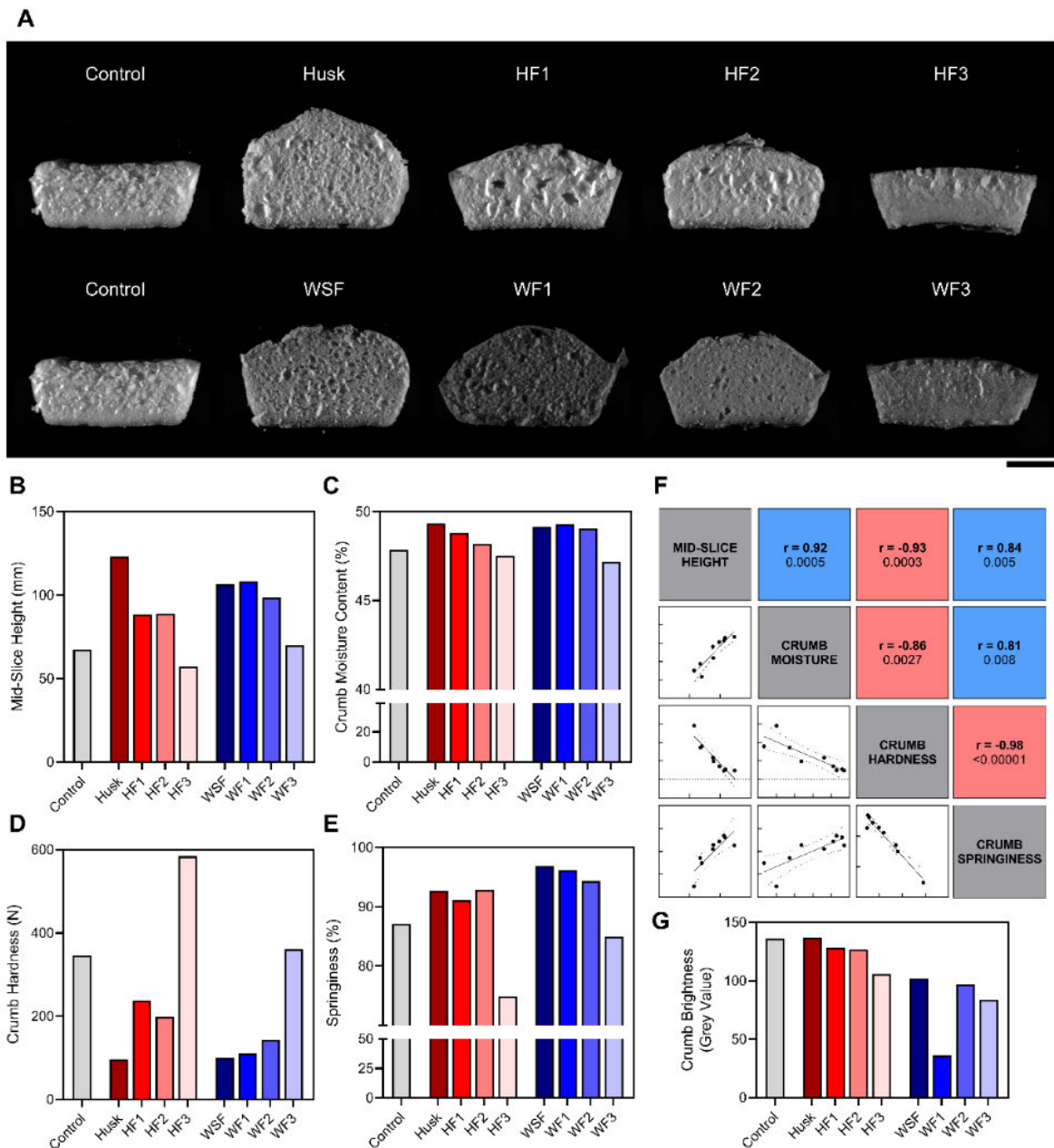
Figure 4-7. Preliminary study on the baking performance of rice flour-based gluten-free breads with P. ovata husk and WSF and their derived fractions used as hydrocolloid replacements. A. Images of bread slices from each loaf. Scale = 5 cm. B. Mid-slice height. C. Crumb moisture content. D-E Texture profile analysis of loaf crumb. F. Correlation matrix of bread properties tested. G. Average crumb colour determined by digital image analysis

Starch + Unfractionated
- Psyllium mucilage fractions are in native assembly anchored to insoluble flour/husk material and might form two partially mixed phases
- Balanced interactions

- Gelatinization temperature ↓
- GF Bread Quality ✓✓✓

Starch + F1
- Little insoluble psyllium material
- Psyllium heteroxylan branching ✓
- The starch matrix is possibly mostly undisrupted
- Psyllium heteroxylan forms balanced homotypic (psyllium-psyllium) and heterotypic interactions (starch-psyllium) improving the viscoelastic properties of the mixture
- No insoluble material to anchor to

- Little effect on gelatinization temperature
- Setback viscosity ↑
- Little effect on starch breakdown
- Strong effects on post-cooking gelling
- GF Bread Quality ✓✓

Starch + F2
- Little insoluble psyllium material
- Psyllium heteroxylan branching ✓✓
- The starch matrix is possibly mostly undisrupted
- Psyllium heteroxylan forms balanced homotypic (psyllium-psyllium) and heterotypic interactions (starch-psyllium) improving the viscoelastic properties of the mixture
- No insoluble material to anchor to

- Little effect on gelatinization temperature
- Setback viscosity ↑
- Little effect on starch breakdown
- Strong effects on post-cooking gelling
- Viscoelastic properties up
- GF Bread Quality ✓✓

Starch + F3
- Abundant insoluble residue
- Psyllium heteroxylan branching ✓✓✓
- Psyllium particles may preferentially self-associate due to the high branching and binding to insoluble particles
- Phase separation may reduce effective water content in the starch phase and promote starch-starch interactions

- Gelatinization temperature ↓
- Phase separation increases sheer forces on starch and lowers the starch phase water content/concentrates the starch which is why viscosity is higher during cooking compared to F1 and F2
- GF Bread Quality ✗

Cooked Rice Flour / Starch Particles

Insoluble Psyllium Particles

Psyllium Heteroxylan

Psyllium WSF / Husk Particles

F1   F2   F3

Starch Continuous Phase

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Figure 4-8. A simplified model summarising the hypothesised interactions and subsequent effects of psyllium husk and WSF and their derived fractions on starch cooking. Yellow and blue backgrounds represent simplified starch and psyllium phases, respectively, and the abruptness of gradient changes correspond to hypothesised phase separation

Supplementary figures
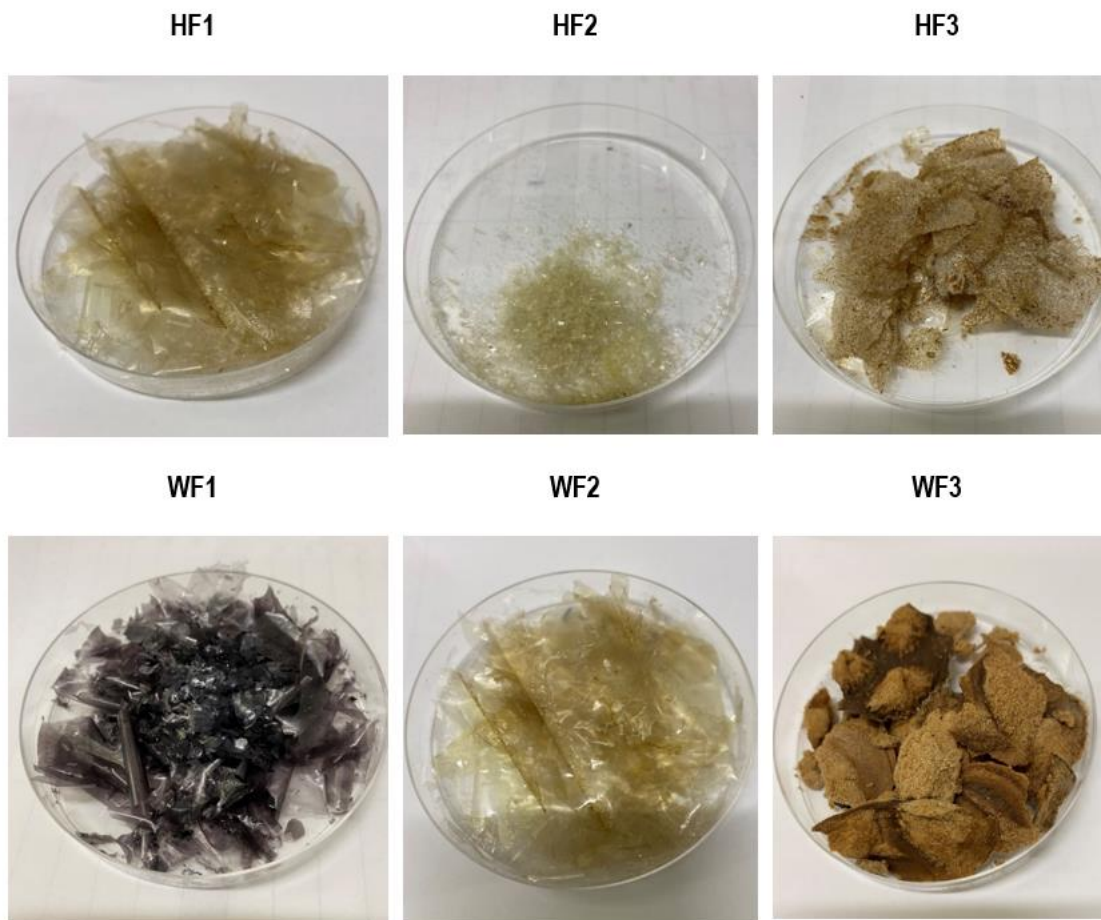
Supplementary Figure 4-1. Appearance of dried mucilage fractions of *P. ovata* husk and *P. ovata* WSF

Acknowledgements

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CHAPTER 5

In vitro Fermentation of diverse *Plantago* seed-derived substrates

Abbreviations

SCFA	Short chain fatty acids
WSF	Whole seed flour
DF	Dietary fibre
POV	*Plantago ovata* whole seed flour
POV-H	*Plantago ovata* husk
POV-S	*Plantago ovata* de-husked seed
Pbel	*Plantago bellidiodes*
Pvar	*Plantago varia*
Ptur	*Plantago turrifera*
Pcun	*Plantago cunninghamii*
Pdeb	*Plantago debilis*
Pmaj	*Plantago major*
DMCV	Dry Matter Cumulative Volume
HCl	Hydrochloric Acid
NH_3	Ammonia
NMR	Nuclear Magnetic Resonance
Brchpn	Branched-chain proportion (compared with total SCFA)
Brch	Total Branched-chain fatty acids

R_{max} Maximum Fermentation Rate

TR_{max} Time at which R_{max} is reached

Sig.   P-value

R_{max} Maximum Fermentation Rate

Introduction

Myxospermy is a process by which seeds of various plant species produce a gel-like layer of mucilage around them when wetted with water (Phan *et al.*, 2020). Psyllium gum, derived from *Plantago ovata* husk, is the one of the most widely used materials from myxospermous species in food (Madgulkar, Rao and Warrier, 2021), due to its viscoelastic properties (Haque and Morris, 1994; Cappa, Lucisano and Mariotti, 2013; Mancebo *et al.*, 2015; Amiri Aghdaei *et al.*, 2012; Fratelli *et al.*, 2018). Due to its high water-holding capacity psyllium husk also serves as a popular dietary fibre supplement which increases stool bulk due to its high water holding capacity and blocks the reabsorption of water in the colon and facilitates their easier passing due to its slippery nature and it is thus used in the treatment of constipation and diarrhoea (Hussain *et al.*, 2016; Washington *et al.*, 1998; Quitadamo *et al.*, 2012).

Psyllium husk is a popular fibre supplement, which increases faecal bulk, but it is quite resistant to fermentation in the human gastrointestinal tract (Marteau *et al.*, 1994; McRorie, 2013; Kaur *et al.*, 2011). This low fermentability, which avoids gas and bloating, makes it a good candidate for fibre supplementation but limits the additional health benefits bestowed by the production of short chain fatty acids (SCFA). In a study where rats were fed with a basal diet supplemented with increasing amounts of psyllium it was found to be partially fermented and about 50% excreted in a highly polymerized form, with a decrease in faecal pH, and an increase in stool bulk (Edwards *et al.*, 1992). Although there was no significant increase in the SCFA concentration in the faeces, the total SCFA output was increased with the increased faecal weight. While the faecal pH of rats fed with the basal diet was 7.59, this reduced to 7.09 and 6.69 with supplementation levels of 15g/kg and 50 g/kg respectively (Edwards *et al.*, 1992). Psyllium husk also displays a delayed onset of gas production compared with other common fibre sources such as wheat bran (Gunn *et al.*, 2020). Marlett and Fischer (2002) attempted fermentation of fractionated psyllium husk, where it was found that the alkali insoluble fraction was not fermentable, the gel-forming fraction was poorly fermented, and the viscous soluble fraction was rapidly and almost completely fermented within a 72-h fermentation period. Psyllium husk in its undigested form

was unable to be fermented by *Bifidobacteria*, but was more fermentable after simulated digestion (Elli *et al.*, 2008). Successful attempts have also been made to increase the fermentation and SCFA production of psyllium husk using ultra-sound, microwave, enzymatic hydrolysis and ball-milling (Hu *et al.*, 2013; Hu *et al.*, 2018; Pollet *et al.*, 2012).

Overall then, in its unmodified form, psyllium husk derived from *Plantago ovata* is clearly poorly fermented, but there may be other members of the *Plantago* genus which can provide different fermentation properties based on their husk polysaccharide profiles. Phan *et al.*, (2016) found differences in the structure and composition of mucilage from seven diverse *Plantago* species, showing that while all were rich in heteroxylan, there were wide differences in its backbone substitution levels between species. Cowley, O'Donovan and Burton, (2021) studied the composition of the whole seed flour of twelve different species of *Plantago* that grow in Australia and found differences in the composition of mucilage as well as in the quantities of mucilage, free sugars, oligosaccharides, protein content, lipid profiles, and mannans in the endosperm. (Leng-Peschlow, 1991) found whole *P. ovata* seeds to be more fermentable than just the husk in a rat feeding trial, producing an increase in faecal butyrate concentrations, which suggests that there may be fermentable components within the seeds of *Plantago ovata* that are not present when the husk is isolated. Cowley, O'Donovan and Burton, (2021) found significant quantities of simple sugars and oligosaccharides including planteose, a galactosyl-sucrose oligosaccharide (Xing *et al.*, 2017) that were identified for the first time in the *Plantago* species by Wattiez and Hanz (1943) but never quantified. Planteose is made up of glucose, galactose and fructose, containing two non-reducing ends with glucopyranose on one end and galactopyranose on the other, which may be fermentable residues. However, to date, there is no research available which examines the *in vitro* or *in vivo* fermentation of planteose, the microorganisms involved nor any fermentation end-products.

Although there are a large number of species of *Plantago* around the world that are used as folk fibre remedies, the literature available on the fermentation of species other than *P. ovata* is limited to *P. asiatica* (Hu *et al.*, 2012; Hu *et al.*, 2018; Hu *et al.*, 2013a; Hu *et al.*, 2013b). There are a number of

Plantago species that are native or naturalized to Australia which are adapted to the climate and therefore with the potential to be produced on a large scale in Australia as a crop. According to the findings of Cowley, O'Donovan and Burton, (2021) the whole seed flours of native *Plantago* are nutritious, containing good ratios of omega-3 and omega-6 fatty acids and protein, along with heteroxylan-rich mucilage with excellent water holding capacity that may prove useful as a stool softener similar to commercial psyllium husk. Extracts of some species such as *P. turrifera* also display good viscoelastic properties in gluten-free food products such as bread (Cowley, 2020). The compositional differences, including variation in the structural features of mucilage of the *Plantago* species identified by Phan *et al* (2016), and the differences in the components of the endosperm included in the whole seed flour of *Plantago* studied by Cowley, O'Donovan and Burton, (2021), could potentially impart different fermentation characteristics for species of native *Plantago.* Also, the inclusion of endosperm components along with the husk of *P. ovata* may alter or improve the fermentability over the use of husk alone from *P. ovata*. However, no studies have been conducted to study the fermentability of the seeds of *Plantago* species other than that of *P. ovata* and *P. asiatica*. Therefore, in this study the fermentability of nine species of *Plantago* from Australia and *Plantago ovata* whole seed flour and fractions was explored.

Materials and Methods

Substrate Preparation

Whole seeds of various species *Plantago bellidiodes* (Pbel), *Plantago varia* (Pvar), *Plantago turriferra* (Ptur), *Plantago cunninghamii* (Pcun), *Plantago debilis* (Pdeb), *Plantago major* (Pmaj) and WOT (an accession of *P. turrifera*), DEW (an accession of *Plantago drummondii*) and LOX (*Plantago* sp., a distinct but unidentified species) were obtained from various sources as mentioned in Supplementary Table 1. The whole seeds were milled to flour using a MM400 mixer mill (Retsch, Germany) (Figure 5-1A).

For preparation of *Plantago ovata* fractions, husk (POV-H) and non-husk tissues (POV-S) were separated by bench-top milling and sieving. Three replicates of 10 g of *P. ovata* whole seeds were milled for 30 s in a spice grinder (BCG200, Breville, Australia). Milled seeds were placed into a fine stainless-steel strainer and the finely powdered husk fraction was separated from the unmilled non-husk tissues by stirring in the strainer for 2 min, changing stirring direction every 20 s. The non-husk tissues were then milled to flour using a MM400 mixer mill (Retsch, Germany). The recovery of husk and seed was recorded (Supplementary Table 2) as an average of 27.5% husk and 72.5% seed. POV was prepared by reconstituting the husk and seed powder in the ratio of 27.5:72.5 (Figure 5-1B). This composite has a similar composition to WSF of *P. ovata* but has been milled separately and re-combined to avoid differences caused due to variation in husk and seed amounts between seed batches.

Wheat bran was included in the experiment as a positive control, while serum bottles containing only medium and inoculum but no substrate, were included as a negative control.

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Dry matter of substrates

The dry matter of each substrate was determined by calculating the percentage of the difference of initial weight and final weight of 0.5g of each substrate oven dried at 105 °C until a constant weight is reached. Dry matter was determined in triplicate for each substrate.

***In vitro* fermentation**

The *in vitro* fermentation technique of Williams et al (2005) was used, as modified by Lu *et al.*, (2020).

In brief: 0.25g of substrate was weighed accurately and recorded, and placed into 60 mL serum bottles with duplicates for each time removal 0h, 10h, 24 h, and five replicates each for the 48 h endpoint for incubation with the human faecal inoculum at 37 °C. An amount of 38 mL of semi-defined, sterile, anaerobic medium was added, prepared as originally described by Lowe *et al.*, (1985), and modified by Williams et al (2005). Cumulative gas production was measured using an AGRS (automated gas recording system-AGRS-III designed College of Animal Science and Technology, China Agricultural University) to directly record the headspace pressure and volume (Yang *et al.,* 2014)(refer Appendix 2 for substrate details for each time removal).

The mixed human faecal inoculum was prepared by pooling faces from seven human volunteers, collected with approval from the Human Research Ethics Approval Committee of the University of Queensland (Approval Number 2017/HE000502). Each donor was self-reported free from gastrointestinal illnesses, age 18+, on an unrestricted diet, and had not taken antibiotics within the three months prior to sample collection. The faeces were pooled and homogenized with 0.9% saline pre-warmed to 37 °C at a dilution ratio of 1:5, followed by filtration with four layers of muslin cloth. To each serum bottle with substrate and medium 2.5 mL of the inoculum was injected and incubated at 37 °C. All inoculation procedures were conducted under a steady stream of CO_2 to maintain anaerobicity, unless otherwise stated. At each time removal the bottles were removed from the incubator and plunged into

an ice-water bath for 20 minutes to retard the microbial activity. Following this the post-fermentation sampling was carried out.

Post fermentation analyses

During the fermentation process, timed removals were done at 0 h, 10 h, 24 h and 48 h. At each removal sampling (3 x 1 mL) from the liquid at the top of the fermentation bottle was made for determination of NH_3 and SCFA. Samples separated for NH_3 analysis were stabilized using 0.2 M HCL, while SCFA samples were stabilized using a protein precipitant (20% metaphosphoric acid and 0.24%4-methylvaleric acid in Milli-Q water) followed by freezing at -20 C until analysis. The remaining contents of the bottle was mixed by manual swirling and transferred to a 50 mL tube for pH measurement and then frozen to be used for polysaccharide analysis.

NH_3 analysis

Samples collected for NH_3 analysis post-fermentation were stabilized using 0.2 M HCl, leading to formation of $NH4^+$ ions. Quantification followed a modified protocol of Baethgen and Alley as reported by Yao *et al.*, (2022). The absorbance reading was recorded at 650 nm using a UV/visible spectrophotometer (Shimadzu UV-1800, Japan) following the reaction of ammonium with sodium nitroprusside and sodium salicylate in a weak alkaline buffer.

SCFA analysis

Samples collected for SCFA analysis were stabilized using a protein precipitant solution. The sample was centrifuged to remove any particles. From the supernatant 700 µL was added to 100 µL, (0.2 mg/mL) Trimethylsilyl propanoic acid sodium salt (TSP) solution in D_2O, present as an internal standard, followed by performing 1 H NMR on a Bruker 700 MHz spectrometer with a 5 mm TCI cryoprobe operating at 298 K. ^{1}H NMR spectra were recorded using a standard excitation sculpting pulse program with an 11 s relaxation delay for 64scans. Seven SCFAs including propionic, n-valeric, n-butyrate,

acetate, iso-valeric and iso-butyric were identified and quantified according to published values . Data was processed using TopSpin 3.6.3 software.

Statistical analysis

All end-product data of fermentation are reported as units per gram of dry matter of starting substrates. The cumulative gas production data was fitted to the monophasic model as described by Groot *et al.*, (1996) as follows:

$$G = (A / (1 + (C/t)^B))$$
Equation 1

Where G is the cumulative gas production (mls) at time t (h); A is the asympototic gas production; B is the switching characteristic of the curve; and C is the time at which half of the asymptotic values has been reached.

In addition, R_{Max} the maximum rate of gas production (mls/h) and T_{RMax} , the time at which it occurs, were calculated according to the following equations reported previously (Williams et al 2005)

$$R_{Max} = (A \times (C^B) \times B \times (T_{Rmax}^{-B-1})) / ((1 + (C^B) \times T_{Rmax}^{-B})^2$$

$$T_{RMax} = C \times (((B-1) / (B+1))^{1/B})$$

The SAS NLIN software was used for curve-fitting. One-way ANOVA was used for significance analyses with Tukey's mean separation using SAS GLM (SAS Institute Inc, Cary, NC, USA, 9.4 Edition, 2016). A P-value of less than 0.05 was regarded as statistically significant.

Correlation analysis was carried out for the mean values of fermentation parameters; total DMCV, acetate, propionate, butyrate, branched SCFA, NH_3, pH at 48 h and mean composition values using SPSS 27 (IBM SPSS Statistics, 2021) software.

Results and discussion

Fermentation kinetics of *Plantago* whole seed flour

Values for the cumulative gas production kinetics from the fermentation of *Plantago* WSF are shown in Table 5-1. The representative fermentation profiles are depicted in Figure 5-2. The fermentation of the *Plantago* WSF, Pdeb yielded the highest cumulative volume of gas per gram of dry matter (DMCV) (48.1 ml g^{-1} DM), which was not significantly different (P>0.05) to DEW, LOX and Pbel but significantly higher than the other five WSF substrates. Pmaj fermentation yielded the lowest DMCV (29.5 ml g^{-1} DM), however it was not significantly different from Ptur, Pvar and WOT. Ptur and WOT, which are different accessions of the species *Plantago turrifera* didn't show any significant difference in their DMCV (Table 5-1). Pbel displayed the fastest R$_{max}$ value of 9.3 mL/h, though it was not significantly different (P>0.05) from DEW, Pdeb, and Pvar. WOT showed the slowest R$_{max}$, although it was not significantly different from LOX, Pcun, Pmaj and Ptur. Ptur took the longest time to reach R$_{max}$ (TR$_{max}$), meaning it is more slowly fermented. However, it was not significantly different (P>0.05) from DEW, LOX, Pbel, Pcun, Pdeb and WOT. Although Pmaj produced the least DMCV, it also had the earliest TR$_{max}$ (3.4h) (Table 5-1), which means it was fermented faster than the other substrates although it produced a lesser amount of gas, suggesting a small quantity of rapidly fermentable substrate was present. However, the TR$_{max}$ of Pmaj was not significantly different from DEW, Pbel, Pcun, Pdeb, Pvar and WOT, suggesting that they all reached R$_{max}$ at a similar time, having a similar lag phase while the rates of gas production were different.

The representative *in vitro* fermentation gas profiles of the *Plantago* WSF are presented in Figure 5-2, which shows that all substrates exhibit minor bi-phasic characteristics with a small initial lag. Similar bi-phasic gas profiles have been observed in the *in vitro* fermentation of insoluble dietary fibre extracted from fruits and vegetables such as banana and celery (Widaningrum *et al.*, 2020).

Compared with wheat bran (the positive control) the onset of the rapid phase of fermentation appeared to be delayed by between 2 and 4 hours (Figure 5-2) which means that all the *Plantago* samples are

fermented more slowly compared to the wheat bran, although ultimately, they produced comparable final gas volumes. (Gunn *et al.*, 2020) observed a similar delay in the onset of fermentation of psyllium compared to wheat bran. This suggests that the human faecal bacteria required a certain amount of time to adapt and adhere to the more complex substrate structure. Potentially the insoluble solid particles from the endosperm could be enveloped by the seed mucilage, which has a high water affinity and could thus form quite a viscous barrier. All the fermentation profiles showed a similar shape (Figure 5-2) although there were differences in the DMCV, R_{max} and TR_{max}, which suggests that there may be a small amount of substrate which is more accessible or more rapidly fermentable. Once this rapidly fermentable fraction is utilized by the bacteria, the remaining substrate would need to be colonized by the bacteria, after which fermentation can continue of those fraction/s which are more resistant to fermentation. For all the *Plantago* substrates, this initial phase was uniformly low in gas production, and it was the second phase where the majority of the gas production occurred until a plateau was reached. Differences in substrate composition may at least in part, be an explanation for differences in fermentabilities of the *Plantago* WSF. The WSF generally comprises a viscous gel within which particles are dispersed. These particles have varying compositions of protein, lipids and oligosaccharides. (Cowley, O'Donovan and Burton, 2021).

End-products of fermented *Plantago* WSF

When bacteria metabolize fermentable carbohydrates they predominantly produce short chain fatty acids such as acetate, propionate and butyrate and other carboxylic acids including lactic acid (Wong *et al.*, 2006; Tan *et al.*, 2014) all of which are known to promote gastrointestinal health (Yang and Zhao, 2021; Martin-Gallausiaux *et al.*, 2021). Acetate is the main SCFA produced through the fermentation of carbohydrates such as xylans and pectin (Macfarlane and Macfarlane, 2006) and can found in venous blood (Wong *et al.*, 2006) following absorption across the gut wall. Propionate is produced mainly through the fermentation of carbohydrates but also some other substrates such as protein (Al-Lahham *et al.*, 2010). Some of this is absorbed into the hepatic portal vein and passed to the liver (Tan *et al.*,

2014) where about 90% of it is metabolized by hepatocytes for processes including gluconeogenesis (Wong *et al.*, 2006).

The production of these three common SCFA, as well as branched-chain SCFA which are products of protein fermentation, are depicted in Table 5-2 and Figure 5-3. The fermentation of Pvar resulted in the highest amount of total SCFA (Table 5-2), which was significantly higher ($P<0.05$) than all other WSF. Although Pdeb had the highest DMCV of all the WSF samples (Table 5-1), its SCFA yield was significantly lower than Pvar. Pearson correlation analysis was carried out between the DMCV and the other fermentation parameters; total SCFA, acetate, propionate, butyrate, branched chain SCFA, NH_3 and pH at 48 h (Supplementary Table 3). The DMCV at 48 h did not display any significant correlation with the total end products at 48. Pvar produced a lower amount of gas yet in comparison to Pdeb, yielded the most SCFA. Therefore, although a WSF might have higher DMCV due to higher fermentability, it does not necessarily mean that it would yield higher amounts of health promoting SCFA compounds. However, Pmaj which had the lowest DMCV (Table 5-1), also yielded the lowest SCFA of all the samples (Table 5-2).

Pvar had the highest production of acetate which was significantly higher than all the other WSF (Table 5-2). When considering SCFA as a percentage of the total, the percentages were in the same decreasing order of acetate, propionate, butyrate and branched SCFA for all substrates. Pvar and Pbel yielded the highest amounts of propionate, while Pmaj and Pvar yielded the highest amounts of branched chain SCFA which are products of protein fermentation (Williams *et al.*, 2019). Pvar had the highest production of total SCFA as well as acetate, however it also produced a large amount of branched SCFA. Pvar has a protein content of 26.01%, which is the highest level of all experimental substrates used here (Table 5-3). The poor gas production, low total SCFA yield, the higher branched chain SCFA (both as a concentration of branched chain SCFA as well as branched chain proportion) suggests that of all the *Plantago* species tested here, Pmaj was the most poorly fermented.

Total SCFA, acetate, propionate, butyrate and branched-chain SCFA increased over the course of fermentation and did not reach a plateau even at 48 h for Ptur, Pbel, Pcun, Pdeb, WOT, DEW, and LOX (Figure 5-3) which all showed very similar patterns of SCFA production over time. For Pvar there was more SCFA production in the first 24 h but from 24–48 h production slowed for all SCFA including acetate, propionate and butyrate, and appeared to begin plateauing. Interestingly, Pmaj, the lowest gas-producing and lowest SCFA producer, displayed a different pattern, where the total SCFA plateaued between 10h to 24 h, followed by an increase in SCFA production between 24 h to 48 h. For most samples the branched SCFA was least amongst all SCFA amounts, however for Pmaj, at 48 h the yield of branched chain SCFA was slightly higher than butyrate, confirming its poor fermentability. The gas production for all substrates reached a plateau before 24 h (Figure 5-2). Despite this, the SCFA production still continued to increase for all samples (Figure 5-3).

The pH change of the substrates did not show any significant difference ($P>0.05$) between the substrates at the end point although there was a decrease in pH over the course of fermentation which acts as an indicator of the fermentation (Table 5-2 and Figure 5-4b). Marteau *et al.*, (1994) also observed a reduction in the faecal pH during dietary supplementation of psyllium in rats.

From 0 h to 10 h there was an increase in the production of NH_3 in all nine substrates as shown in Figure 5-4a. During 10h to 24 h, the NH_3 production plateaued in Ptur, Pvar, WOT, Pcun, Pmaj, Pdeb and LOX, followed by an increase from 24 h to 48 h However, for DEW, the NH_3 production increased in the first 10h followed by a plateau until 48h. Pbel on the other hand, showed increasing NH_3 production until 24 h, after which a plateau was reached. The increase in NH_3 from 0 h to 10 h could indicate the lack of easily fermentable carbohydrates and also that the bacteria in the inoculum take time to adapt to the substrate which appeared as a viscous, gel-like substrate settled at the bottom of the fermentation vessel without mixing with the medium. The lack of fermentable carbohydrates could cause the bacteria to ferment protein materials such a dead bacteria, protein found in the inoculum, or any peptides and amino acids within the medium. In this case, the substrates had a significant protein content ranging from 13.80% to 26.01% (w/w) (Table 5-3). Correlation analysis showed a positive

correlation (Pearson r = 0.801, sig = 0.03) (Supplementary Table 4) between the protein content and ammonia production. Although Pvar yielded the highest SCFA of all substrates, it also had the highest protein content which led to it showing the highest NH_3 production (Table 5-2 and Figure 5-4a.) Plateauing of NH_3 production in most substrates between 10 h to 24 h could mean that the bacteria were adapted to the environment and began metabolizing any fermentable carbohydrates in the medium. The increase in NH_3 production after 24 h could mean that the fermentable carbohydrate material was depleted and the difficulty of utilizing the remaining complex polysaccharides stimulated a return to protein fermentation.

Fermentation of proteins within the medium leads to their breakdown into amino acids which are further converted to branched chain SCFA, ammonia, amines, phenols and indoles, especially when the bacteria involved in fermentation lack access to fermentable carbohydrates in the medium (Rowland *et al.*, 2018). Increased protein fermentation is considered harmful to the health of the gastrointestinal tract (Williams, Vertegen and Taminga, 2001) as products of protein fermentation such as amines, indoles, sulphides, thiols and phenols are potentially toxic (Rowland *et al.*, 2018; Macfarlane and Macfarlane, 2012). Excessive amounts of toxic metabolites from protein fermentation have been associated with gastrointestinal diseases such as colon cancers, bowel diseases and Crohn's disease (Bingham 1998; Bingham 1999; Liu *et al.*, 2015). However, psyllium is usually consumed in small quantities as a hydrocolloid component in processed food or as a fibre supplement which is unlikely to create a negative health impact due to protein fermentation. Also, the samples used in this study are not pre-digested which led to a higher protein availability in the medium. However, protein digestion in the upper GI tract before *in vivo* fermentation or pre-digestion prior to *in vitro* fermentation would reduce the amount of protein from the WSF in the medium thereby reducing the protein fermentation.

Comparison of *P. ovata* husk, seed and whole seed flour

The native *Plantago* species used in this experiment have seeds which are much smaller than *Plantago ovata*, the source of psyllium husk which is widely used in food and fibre. Milling into flour is therefore

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more convenient on a whole seed basis since extraction of a husk fraction is a lot more laborious (Cowley, 2020; Cowley, O'Donovan and Burton, 2021). Therefore, use of the native *Plantago* species involves the fermentation of the mucilage fraction as well as the endosperm components such as protein, fats, sugars etc *(Table 5-3). This made it impossible determine the fermentability of the seed and husk components separately. In a rat feeding trial, psyllium husk supplementation led to an increase in the stool weight, SCFA output and a decrease of the faecal pH. It was partially fermented and about 50% of the consumed psyllium husk was excreted (Edwards *et al.*, 1992). Psyllium husk mucilage consists of mucilage fractions which are considered rapidly fermentable, poorly fermentable and non-fermentable (Marlett and Fischer, 2002; Marlett and Fischer, 2003). Psyllium husk was also unable to be metabolized by *Bifidobacteria* in its native form, but could be partially metabolized after simulated gastric digestion (Elli *et al.*, 2008).

In this experiment a preliminary attempt was made to study fermentation of the easily-separable husk (containing mucilage) and seed (endosperm and embryo) of *P. ovata* in comparison with the WSF. Table 4 and Figure 5 shows the fermentation kinetics of the *P. ovata* WSF (POV) and the husk and seed fractions abbreviated as POV-H and POV-S, respectively. The DMCV (Table 5-4) of POV-S was the highest and was significantly higher ($P<0.05$) than POV-H. A DMCV value was calculated by using the DMCV values of POV-H and POV-S considering their relative proportion present in POV. The calculated DMCV (47.6 mL/g DM) was similar to the actual DMCV of POV (47.7 mL/ g DM). The R_{max} was comparable in all substrates. The TR_{max} occurred significantly earlier for POV-H compared to POV-S. The TR_{max} of POV was not significantly different from the POV-S. The onset of gas production of all three substrates was slower in comparison to wheat bran (Figure 5-5). Gunn *et al.*, (2020) made a similar observation with husk, but our findings show that the seed of POV (POV-S) is also slowly fermented compared to wheat bran. Both POV-H and POV showed a similar gas profile, but POV-H showed a different profile with a clear biphasic profile. The fermentation end-products of POV and fractions are shown in Table 5-5 and Figure 5-5. There was no significant difference between substrate (Figure 5-5a,b) for total SCFA and acetate concentrations ($P>0.05$). However, the POV-H produced

more propionate compared to POV-S and POV-H (P<0.05) with POV-H>POV>POV (Table 5-5 and Figure 5-5a). Similarly, psyllium husk supplementation increased the SCFA in faeces as well as the molar proportions of acetate and propionate during a human psyllium husk feeding trial (Edwards *et al.*,1992).

The production of butyric acid was significantly different between all three substrates where the highest butyrate production was in POV-S with POV-S>POV>POV-H (Table 5-5 and Figure 5-5a). Fermentation of POV-S produced the highest branched SCFA concentration which was significantly higher than the other two substrates (P<0.05). The variation of propionate/total SCFA% and the butyrate/total SCFA%, also showed a similar pattern to the propionate and butyrate respectively as shown in Figure 5-5b. This suggests that the husk/mucilage components promoted the production of propionate, while the seed endosperm/embryo contents promoted the production of butyrate. In a 4-week feeding trial of rats with *P. ovata* seeds (comparable to the POV substrate used here) or husk (comparable to the POV-H substrate used here), there was an increase in faecal bulk and metabolic effects, and an increase in the total acetate production in the faeces and caecal contents of the rats, suggesting that the seeds are partly fermentable. However, it was not able to differentiate between the effects of the husk and seeds as the endosperm/embryo was not separated from the husk and tested (Leng-Peschlow, 1991). A similar observation was made in a randomized human pre-clinical feeding trial. Fernández-Bañares *et al.*,(1999) observed an increase in the butyrate production upon feeding of *P. ovata* seeds which matched the observation here of high butyrate production from the seed. The POV-S which includes the seed endosperm which contains the vast majority of seed protein, produced the highest NH_3 (Table 5-5). This agreed with the positive correlation of protein content and NH_3 production observed in the native *Plantago* WSF (Supplementary Table 4). At the end of fermentation, the pH of the three substrates were not significantly different (P>0.05).

Correlations between *Plantago* composition and fermentation parameters

Means of compositional data of the native *Plantago* WSF (Pmaj, Ptur, Pcun, Pdeb, Pbel, Pvar and Pov) were obtained from Cowley (2021), and monosaccharide data for WOT, DEW and LOX determined in this study are shown in Table 5-3. The mucilage characteristics, proximate composition, free sugars and oligosaccharides for WOT, DEW and LOX couldn't be obtained during the period of this project due to time and equipment constraints. The available compositional data were correlated with the fermentation parameters as shown in Supplementary Table 4. A strong positive correlation (Pearson r = 0.801, sig = 0.03) was observed between NH_3 and protein. A higher NH_3 production was observed for Pvar, the native WSF with the highest protein content and also a higher NH_3 production was seen in the protein-rich POV and POV-S fraction fermentations, as discussed earlier. Strong negative correlations were observed between the fat content and total SCFA (r = -0.759, sig. = 0.048) and acetate (r= -0.797, sig. =0.032). As acetate is usually the largest component of total SCFA, the fact that both of these parameters were correlated to one compositional parameter is justified. The DF content was negatively correlated to the branched SCFA. The increased DF suggests the presence of more fermentable carbohydrates, which would reduce the protein fermentation, resulting in lower concentrations of branched SCFA. Arabinose was positively correlated to the total SCFA, acetate and propionate, which could mean that arabinose side chains in the mucilage heteroxylan are being cleaved and metabolised to produce SCFA, particularly acetate and propionate. The relationship between propionate and arabinose is interesting because during the fermentation of *P. ovata* substrates POV, POV-H and POV-S (Table 5-5 and Figure 5-5), the POV-S containing less arabinose than the POV-H, produced the lowest amount of propionate, while the POV-H, which is rich in heteroxylan with high amounts of arabinose and xylose, produced higher amounts of propionate.

Table 4 and Figure 5 shows the fermentation kinetics of the *P. ovata* (POV) WSF and husk and seed fractions abbreviated as POV-H and POV-S, respectively. The DMCV (Table 5-4) of POV-S was the highest, being significantly higher (P<0.05) than POV-H but not significantly different (P>0.05) to the POV. However, the R_{max} was similar in all substrates. The TR_{max} was lowest for the POV-H but it was

not significantly different (P>0.05) from POV. The TR$_{max}$ of whole POV was not significantly different from the POV-S. The onset of gas production of all three substrates was slower in comparison to wheat bran (Figure 5-5). Planteose is an oligosaccharide found in the *Plantago* WSF which showed a strong correlation with total SCFA, acetate and butyrate concentrations. Planteose is mainly found in the endosperm of the *Plantago* seeds, and this could be the reason for the higher production of butyrate by POV-S compared to the POV-H (Figure 5-5 and Table 5-5). Planteose is an oligosaccharide which was first identified in the *Plantago* species by Wattiez and Hans, (1943) and later identified in 35 species of *Plantago* (Gorenflat and Bourdu, 1962). It has also been found in plants such as sesame, tobacco , fermented cocoa beans (Hatanaka, 1959) and in the mucilage of chia seeds (Xing *et al.*, 2017). It has been found to be broken down by OmAGAL2 from the weed *Orobanche minor* during the germination process to release hexoses (Okazawa *et al.*, 2022). Raffinose is a similar oligosaccharide made of the same components, however while raffinose has galactose attached to the glucose residue in sucrose, planteose has the galactose forming a glycosidic linkage with the fructose end of sucrose (Daude *et al.*, 2012). It is a galactosyl-sucrose oligosaccharide consisting of glucose, fructose and galactose, with two non-reducing ends with glucopyranose on one end and galactopyranose on the other (Xing *et al.*, 2017). However, there is no research available on the fermentation of planteose, including the microorganisms involved, or the fermentation products. This study is the first to report on the fermentability of planteose within *Plantago*. Planteose content of the WSF was positively correlated to the content of raffinose and the galactose in the monosaccharides as shown in Table 5-5. Galactose is a sugar component of planteose (Xing *et al.*, 2017).

Conclusions and future directions

Fermentation of different *P. ovata* substrates and native *Plantago* WSF led to the production of SCFA, however the 10 species of *Plantago* used in this study were not overall very well fermented, similar to previously published reports. It appears that the seed component promotes the production of butyrate and acetate, while the heteroxylan-rich mucilage promotes the production of propionate and acetate. The composition of the whole seed flour had a significant impact on their fermentability. An increasing fat content was correlated with a reduction of SCFA production, a higher protein content was associated with an increase in ammonia production, and a higher DF content (mainly rhamnose and mannose contents) led to lower branched-chain SCFA. The poorly studied oligosaccharide planteose was found to be a likely fermentation candidate with amount correlating strongly with the production of linear SCFAs. It can be concluded that similar to *P. ovata*, the native *Plantago* species studied here are also poorly fermentable, and some, like *Plantago major,* are even less fermentable than *P. ovata* itself.

The complex nature of the heteroxylan and the high degree of branching may be limiting bacterial activity. However, there were certain limitations in this experiment. The compositional data for DEW, LOX and WOT could not be obtained within the duration of this project. It is suggested that a compositional analysis of the aforementioned species should be carried out in order to make this study publishable. Another suggestion would be the fermentation of pure planteose to study its degree of fermentation, the bacteria involved in the fermentation and the fermentation end-products. *P. ovata* husk and seed showed differences in their gas production and fermentation end-products so similar studies could be carried out using mucilage extracted from the native *Plantago* and de-mucilaged seeds to confirm if the observations of *P. ovata* fractions is relevant to other species of *Plantago*. In future studies *Plantago* species grown under the same conditions could be used to eliminate any growth and environmental effects leading to differences between the fermentability of substrates. The results of this study suggest that the whole *P. ovata* seed and the de-husked seed are more fermentable than the husk alone. Therefore, it is possible that fermentation of whole *P. ovata* seed may lead to better

gastrointestinal benefits *in vivo,* with the faecal bulking action of the husk combined with the added benefit of improved fermentability, compared to consuming psyllium husk alone. However, it would require *in vivo* studies to confirm any improved gastrointestinal outcomes. The fermentation of native and naturalised *Plantago* from Australia has shown them to be of limited fermentability , similar to *P. ovata,* although they have high water holding capacity. Therefore, they may be good candidates for use in the food and fibre industry, similar to the current uses of psyllium husk. In their powdered form they could be used as dietary fibre supplements, targeted at providing bulking effects, or for increasing the ease of faecal elimination. Native *Plantago* WSF could also be included in high fibre formulated food products. In conclusion, we suggest Australian native *Plantago* whole seed flours, which are already adapted to the climatic conditions and can be grown in Australia in food and fibre uses, as substitutes for imported psyllium husk.

Acknowledgements

Figures

a) Native *Plantago* Whole → Milling → Native *Plantago* WSF

b) *P. ovata* whole seed → Grinding → Husk and seed separated → Milling → Seed (POV_S) and husk (POV_H) powder → Mixing → *P. ovata* WSF (POV)
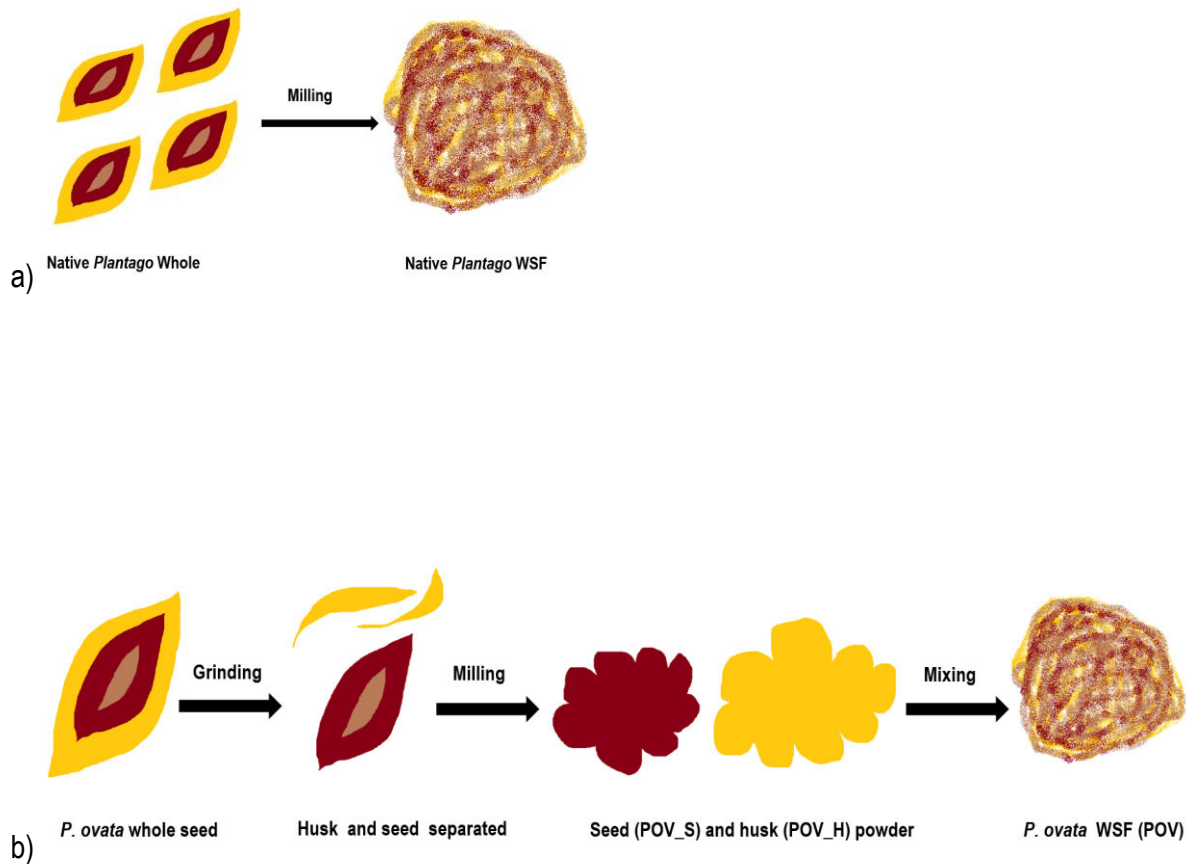
Figure 5-1. Preparation of *Plantago* substrates for fermentation. **a)** Preparation of *Plantago* WSF **b)** Preparation of POV-H, POV-S, and POV.

Table 5-1. Gas production kinetics of fermentation of *Plantago* WSF.

Substrate	Cumvol (mL)	DMCV (mL/g DM)	A1 (mL/g DM)	B1	C1 (h)	TRmax (h)	Rmax (mL/h)
DEW	9.5abc	41.1ab	44.8ab	3.8a	5.4bcde	4.7abc	8.5ab
LOX	8.8abcd	39.0ab	40.1bc	3.1a	6.9a	5.5ab	5.1c
Pbel	9.8ab	42.0ab	44.3ab	4.0a	5.1dec	4.4abc	9.3a
Pcun	8.3bcd	36.6bc	36.5bc	3.5a	6.3abc	5.2ab	5.6c
Pdeb	10.9a	48.1a	53.1a	3.3a	5.7abcd	4.7abc	8.5ab
Pmaj	6.8d	29.5c	31.3c	3.3a	4.2e	3.4c	6.7bc
Ptur	7.5dc	33.0bc	33.6c	3.9a	6.8ab	5.8a	5.2c
Pvar	7.9bdc	35.6bc	35.3bc	4.4a	4.5de	4.1bc	9.0ab
WOT	7.6dc	34.8bc	35.2bc	3.1a	6.3abc	4.8abc	4.9c
Prob.	*<.0001*	*<.0001*	*<.0001*	*0.0849*	*<.0001*	*0.0004*	*<.0001*
MSD	*2.18*	*9.39*	*9.76*	*1.59*	*1.47*	*1.60*	*2.42*

[a,b,c,d,e] Superscripts which differ within the same column are significantly different from each other (P < 0.05).

A is the asymptotic gas production (mL/ g DM), B is the switching characteristic of curve, C is the time at which half of the asymptote has been reached (T1/2), Rmax is the maximum fermentation rate and TRmax is the time at which Rmax was reached. MSD is the Minimum Significant difference.

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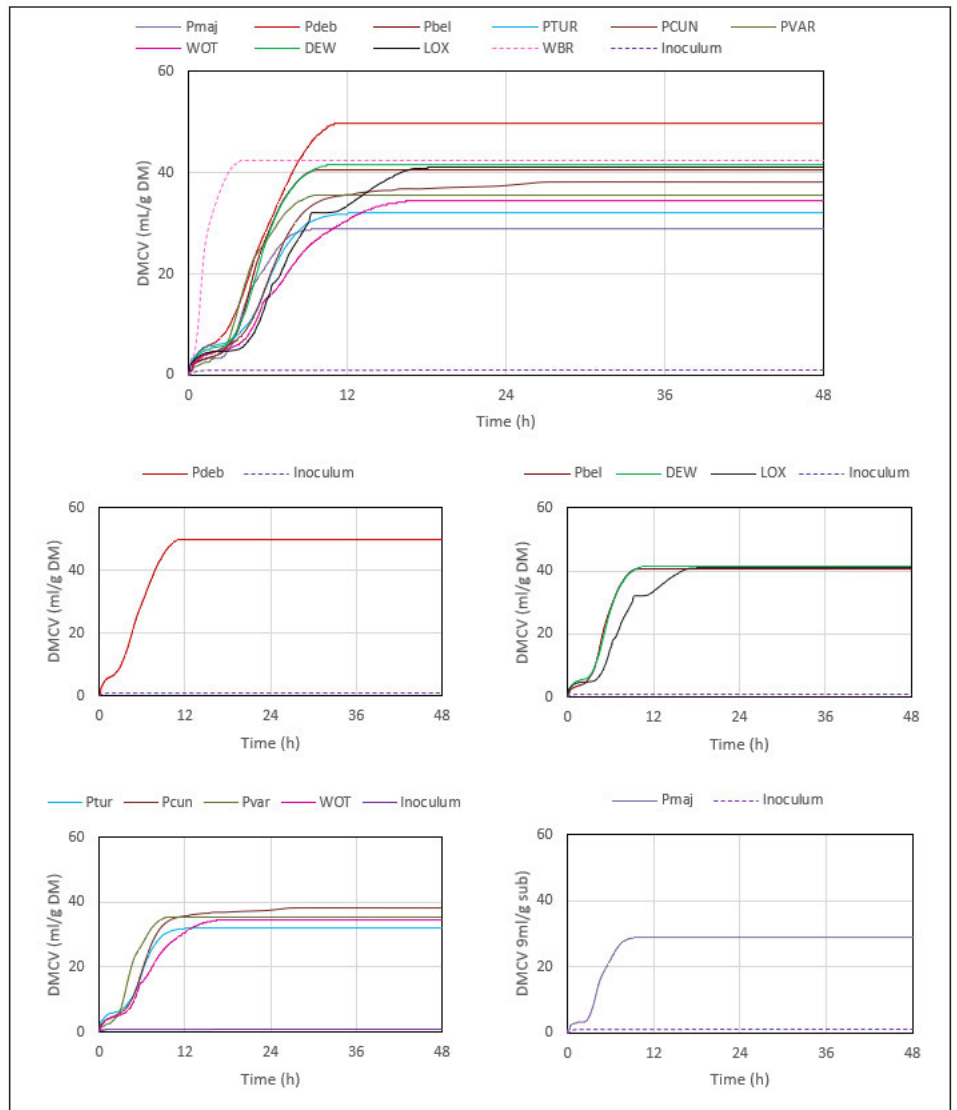
Figure 5-2. Representative cumulative gas profiles during *in-vitro* fermentation of *Plantago*

Table 5-2. End-products from *in vitro* fermentation of Whole *Plantago* substrates only.

Substrate	n	Acetic (mmoles/g DM)	Propionic (mmoles/g DM)	Butyric (mmoles/g DM)	Branched-Chain (mmoles/g DM)	Total (mmoles/g DM)	Brchpn	% of total SCFA			NH3 (mmoles/g DM)	pH
								Acetate	Propionate	Butyrate		
DEW	11	2.6b	0.8bc	0.39bc	0.29b	4.1bc	0.069b	64.4a	20.0ab	8.7b	1.9c	6.73a
LOX	11	2.5b	0.7c	0.46ab	0.29b	4.0bc	0.077b	63.7ab	18.6dc	10.1ab	2.0bc	6.72a
Pbel	11	2.7b	0.9a	0.45abc	0.32b	4.4b	0.075b	61.6c	20.9a	10.0ab	2.0bc	6.68a
Pcun	11	2.5b	0.8bc	0.502a	0.30b	4.1bc	0.077b	61.6c	19.2bcd	11.5a	2.1bc	6.70a
Pdeb	11	2.6b	0.8bc	0.50ab	0.31b	4.2b	0.078b	62.1bc	19.2bcd	10.8a	1.9c	6.67a
Pmaj	11	2.3b	0.6d	0.34c	0.38a	3.7c	0.103a	63.1abc	16.9e	9.8ab	1.8c	6.57a
Ptur	11	2.6b	0.8b	0.44abc	0.29b	4.1bc	0.072b	62.7abc	19.8abc	10.3ab	2.4ab	6.72a
Pvar	11	3.1a	1.0a	0.48ab	0.38a	4.9a	0.080b	63.5ab	19.7abc	8.8b	2.6a	6.70a
WOT	11	2.6b	0.8bc	0.49ab	0.31b	4.1bc	0.078b	63.8ab	18.0de	10.4ab	2.0bc	6.73a
MSD		0.34	0.09	0.113	0.058	0.50	0.0115	1.84	1.29	1.83	0.49	0.181
Prob.		<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	<.0001	0.1464
Removal		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0006
Sub x Removal		0.0383	<.0001	0.0630	0.0019	0.0117	<.0001	<.0001	<.0001	<.0001	0.2632	0.0181

a,b,c,d Superscripts which differ within the same column are significantly different from each other (P < 0.05).
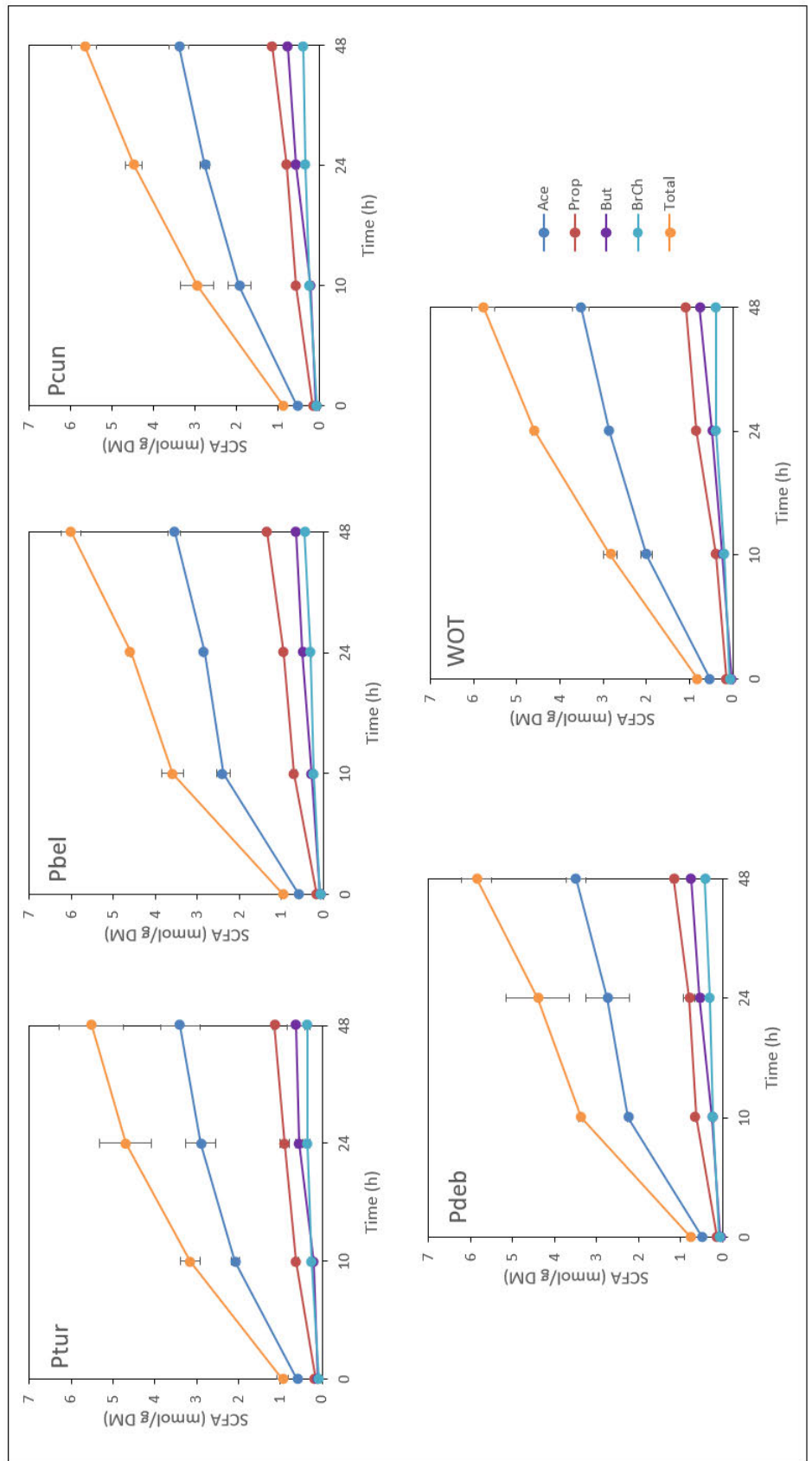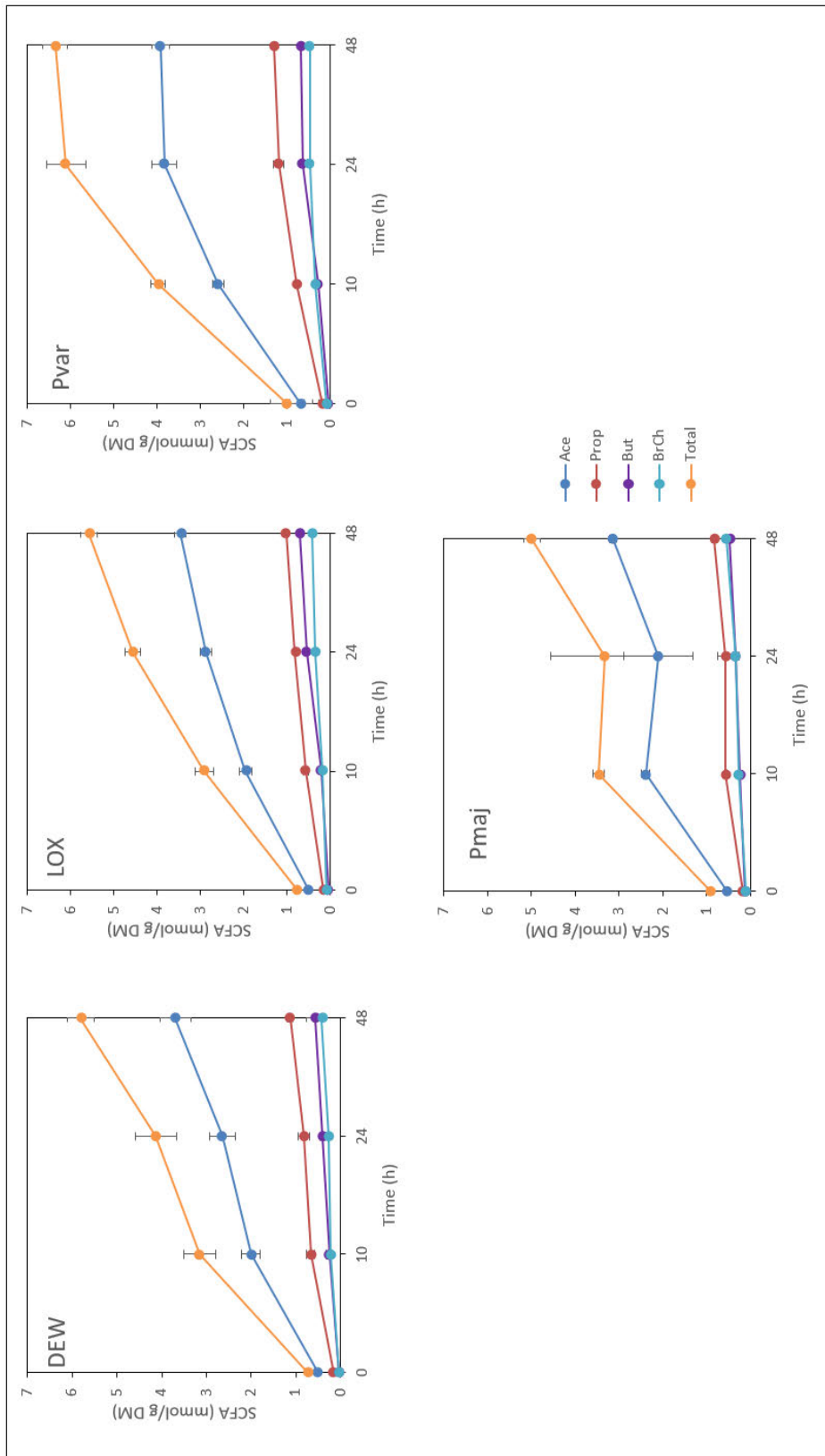
Figure 5-3. SCFA production in time, of *Plantago*; Total SCFA, acetate, propionate, butyrate, branched chain SCFA. Data are expressed as means while the error bars indicate standard deviation.

a.

b.
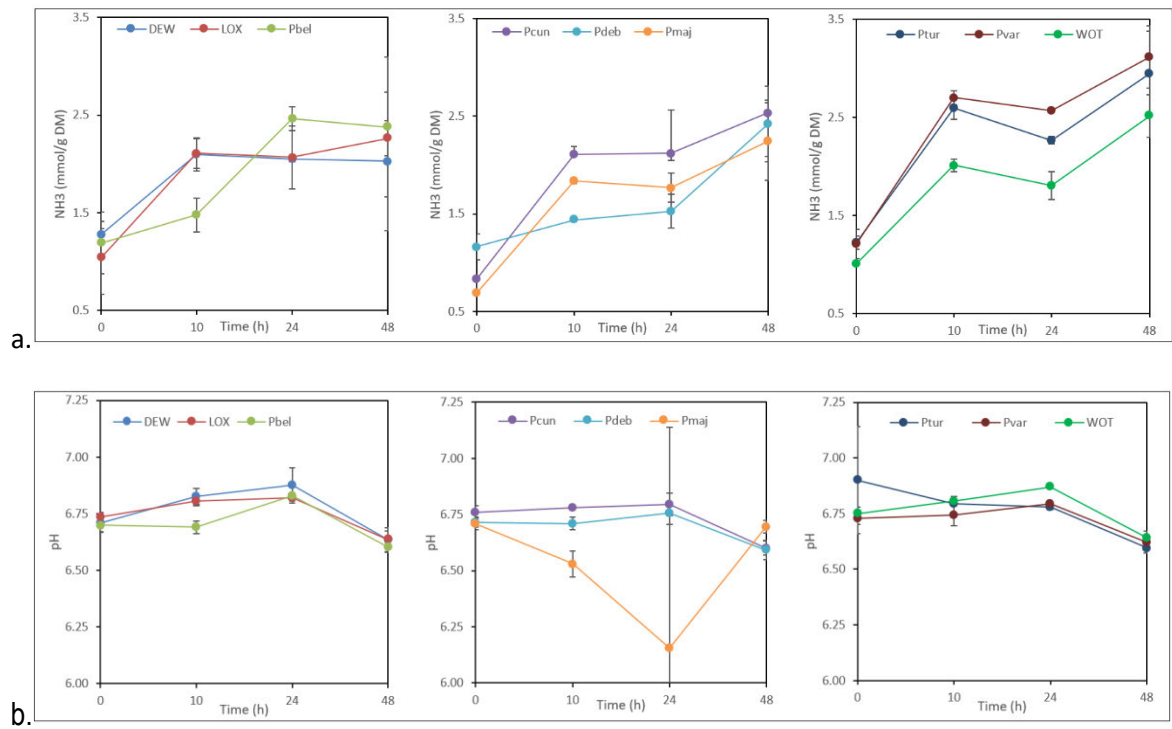
Figure 5-4. a) NH$_3$ production of *Plantago*. Data are expressed as mean standard deviation. b) pH changes of Plantago. Data are expressed as the mean, and the error bars show the standard deviation.

Table 5-3. Mean composition of *Plantago* WSF and mean fermentation parameters at 48 h *in vitro* fermentation.

		Pma[b]	Ptur[b]	LOX[a]	Pcur[b]	WOT[a]	DEW[a]	Pdeb[b]	Pbel[b]	Pvar[a]	Pov[b]
Fermentation parameters	Total SCFA (mmol/g DM) at 48h	4.99	5.52	5.58	5.68	5.78	5.81	5.85	6.01	6.36	6.5
	DMCV (mL/g DM) at 48h	29.538	32.929	41.18	36.611	34.44	41.58	48.094	41.963	34.584	47.677
	NH3 (mmol/g DM) at 48h	2.24	2.95	2.26	2.53	2.52	2.03	2.42	2.37	3.12	1.5
Monosaccharides composition	Rhamnose	0.7	1.88	1.92	3.19	2.18	2.04	2.09	2.45	1.55	1.22
	Mannose	11.84	18.67	16.58	15.35	14.27	13.77	18.18	12.02	11.16	12.67
	Galacturonic acid	0.56	1.19	0.75	1.15	1.58	1.53	0.76	1.49	0.99	1.08
	Glucose	5.98	3.01	2.34	2.83	3.7	4.42	3.97	2.74	3.67	2.35
	Galactose	2.17	1.92	2.26	1.5	3.19	3.09	3.06	2.22	3.12	3.4
	Xylose	8.85	14.05	6.54	16.15	11.44	7.66	8.23	13.02	9.93	18.83
	Arabinose	3.44	7.64	5.42	8.32	8.02	10.97	6.11	6.87	6.93	11.86
	A/X	0.39	0.54	0.83	0.52	0.70	1.43	0.74	0.53	0.7	0.63
Mucilage characters	Water absorption capacity% (per gram of whole seed)	12.77	22.2	NA	12.38	NA	NA	6.33	20.38	6.58	13.08
	Mucilage yield%(per gram of whole seed)	14.62	18	NA	19.52	NA	NA	14.61	17.19	13.96	23.84
Proximate composition	Carbohydrates as simple sugars%	3.28	2.63	NA	2.98	NA	NA	2.67	2.53	3.31	2.91
	Dietary fibre%	34.19	48.36		48.49			42.39	40.8	37.34	51.41
	Protein%	16.36	18.03		19.53			13.8	20.43	26.01	11.42
	Fat%	12.7	11.51		13.14			12.9	12.07	9.11	7.08
Free sugars and oligosaccharides (μg/mg)	Glucose	3.34	0.5	NA	0.4	NA	NA	0.1	0.19	0.49	0.11
	Fructose	0.27	0.01	NA	1.43	NA	NA	0	0.04	0.05	0.9
	Sucrose	11.14	7.93	NA	6.14	NA	NA	5.55	2.9	7.24	3.02
	Planteose	17.95	21.22	NA	16.71	NA	NA	20.97	22.08	25.2	25.65
	Raffinose	0.08	0.08	NA	0.04	NA	NA	0.13	0.11	0.12	0.17
	Stachyose	0.004	0.011	NA	0.26	NA	NA	0.003	0.001	0.001	0.016

The increasing colour intensity of DMCV (pink), NH3 (blue) and total SCFA (green) indicate increasing mean values for each parameter. a=this study, b=Cowley (2021), NA = data not available

Table 5-4. Gas production kinetics of fermentation of fractionated *Plantago ovata*.

	Cumvol (mL)	DMCV (mL/g DM)	A1 (mL/g DM)	B1	C1 (h)	TRmax (h)	Rmax (mL/h)
Fraction							
POV-H	8.9[b]	38.1[b]	45.8[a]	1.1[b]	8.6[a]	0.5[b]	5.1[a]
POV-S	11.50[a]	51.2[a]	55.2[a]	2.2[a]	8.1[a]	4.5[a]	5.4[a]
POV	11.5[a]	47.7[a]	54.7[a]	1.7[ab]	10.9[a]	3.3[ab]	4.8[a]
Prob.	0.014	0.011	0.256	0.028	0.451	0.029	0.697
MSD	2.05	9.43	15.70	0.94	6.41	3.11	2.31

[a,b,c,d,e] Superscripts which differ within the same column are significantly different from each other ($P < 0.05$).

A is the asymptotic gas production (mL/ g DM), B is the switching characteristic of curve, C is the time at which half of the asymptote has been reached (T1/2), Rmax is the maximum fermentation rate and TRmax is the time at which Rmax was reached. MSD is the Minimum Significant difference.

Table 5-5. End-products from *in vitro* fermentation of Whole *Plantago ovata*.

Fraction	Acetic (mmoles/g DM)	Propionic (mmoles/g DM)	Butyric (mmoles/g DM)	Branched-Chain (mmoles/g DM)	Total (mmoles/g DM)	Brchpn	% of total SCFA			NH3 (mmoles/g DM)	pH
							Acetate	Propionate	Butyrate		
POV-H	2.8[a]	1.2[a]	0.3[c]	0.28[c]	4.5[a]	0.068[b]	62.3[a]	24.6[a]	6.3[c]	1.5[b]	6.63[a]
POV-S	2.8[a]	0.9[b]	0.7[a]	0.38[a]	4.8[a]	0.080[a]	59.9[a]	19.5[c]	12.6[a]	2.1[a]	6.64[a]
POV	2.7[a]	1.0[b]	0.5[b]	0.32[b]	4.5[a]	0.079[a]	61.0[a]	20.9[b]	10.1[b]	1.6[b]	6.66[a]
MSD	0.24	0.09	0.06	0.0263	0.33	0.0071	1.49	1.31	0.94	0.33	0.06
Prob. Fraction	0.7230	<.0001	<.0001	<.0001	0.0536	0.0003	0.0019	<.0001	<.0001	0.0005	0.6229
Removal											
0	0.5[d]	0.1[d]	0.1[d]	0.1[d]	0.7[d]	0.094[a]	64.7[a]	19.4[b]	6.4[b]	1.4[b]	6.71[a]
10	2.0[c]	0.6[c]	0.2[c]	0.3[c]	3.1[c]	0.083[b]	64.6[a]	20.2[b]	6.9[b]	2.2[a]	6.59[b]
24	3.3[b]	1.1[b]	0.6[b]	0.4[b]	5.3[b]	0.067[c]	61.7[b]	20.6[b]	11.0[a]	1.6[b]	6.79[a]
48	3.8[a]	1.5[a]	0.0[a]	0.4[a]	6.5[a]	0.069[c]	58.0[c]	23.5[a]	11.6[a]	1.7[b]	6.58[a]
MSD	0.33	0.13	0.08	0.04	0.45	0.01	2.06	1.81	1.30	0.46	0.09
Prob. Removal	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0009	<.0001
Prob. Sub x Removal	0.0205	<.0001	<.0001	<.0001	0.0360	<.0001	0.0007	<.0001	<.0001	0.1709	0.4274

a,b,c,d Superscripts which differ within the same column are significantly different from each other (P < 0.05).

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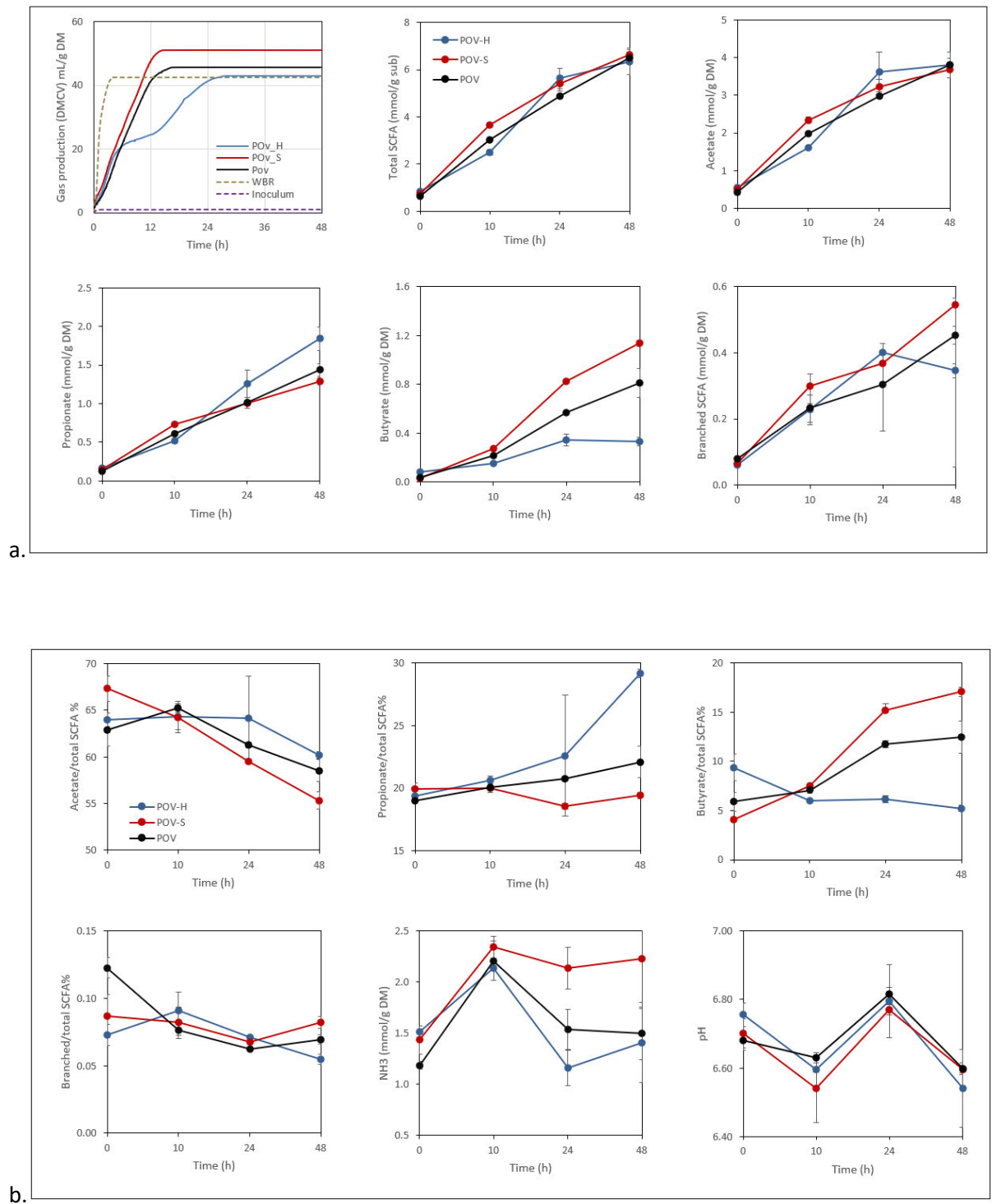
Figure 5-5. *In vitro* fermentation of *Plantago ovata* fractions: Gas production, SCFA concentrations, SCFA%, NH₃ and pH variation with time. Data are expressed as mean standard deviation.

Supplementary Figures

Supplementary Table 5-1. Sources for all *Plantago* species used in this study.

Accession Name	Species Identity		Origin	Seedbank	Voucher Number	ITS Genbank Accession No.
Pov	*P. ovata*	P	Austral Herbs, NSW, Australia	-	JMC006	MW139238
Pmaj	*P. major*	P	Organics Australia, Australia	-	JMC005	MW139237
Pcun	*P. cunninghamii*	R	St. George, Queensland, Australia	-	JMC002	MW139233
Ptur	*P. turriferra*	C	Loxton, South Australia, Australia	-	JMC007	MW139241
Pdeb	*P. debilis*	R	Charlwood, Queensland, Australia	-	JMC003	MW139234
Pbel	*P. bellidioides*	S	Petal Point, Tasmania, Australia	TSCC	0008349-a	MW139231
Pvar	*P. varia*	R	Aranda Bushland, Australian Capital Territory, Australia	-	JMC008	MW139242
WOT	*P. turriferra*	C	Hattah, Victoria, Australia	-	JMC012	-
LOX	*P. sp.*	C	Loxton, South Australia, Australia	-	JMC011	-
DEW	*P. drummondii*	C	Red Cliffs, Victoria, Australia	-	JMC013	-

Single letters denote material origin: P, purchased; C, private land collected; R, collected and donated by researcher; S, seedbank withdrawal.
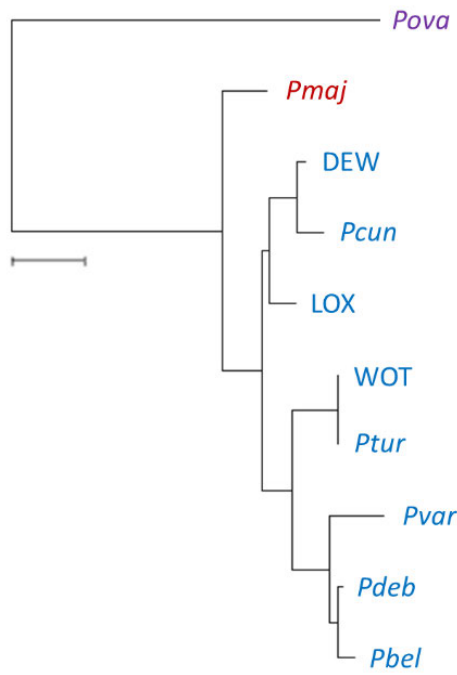
Supplementary Figure 5-1. Maximum likelihood tree of nuclear ribosomal DNA internal transcribed spacer (ITS) regions from the *Plantago* accessions studied here. Scale = 0.01 substitutions per site. Purple = native to central Asia, Red = native to western Asia, Blue = native to Australia.

Supplementary Table 5-2. Husk (POV-H) and seed (POV-S) recovery from whole P. ovata seed milling.

From 10g	Husk (g)	Seed (g)	Sum (g)	Loss (g)	%	Husk (POV-H)	Seed (POV-S)	Recovery	Loss
R1	2.85	7.06	9.91	0.09	R1	28.5	70.6	99.1	0.9
R2	2.9	7.03	9.93	0.07	R2	29.0	70.3	99.3	0.7
R3	2.43	7.45	9.88	0.12	R3	24.3	74.5	98.8	1.2
AVG	2.73	7.18	9.91	0.093333	AVG	27.3	71.8	99.1	0.9
SD	0.26	0.23	0.03	0.025166	SD	2.6	2.3	0.3	0.3

Three replicates of 10g were used for milling, husk and seed percentages were calculated.

Supplementary Table 5-3. Correlations between fermentation products of *Plantago*.

		Acetate	Propionate	Butyrate	Total SCFA	Acetate/ total SCFA%	Propionate/ total SCFA%	Butyrate/ total SCFA%	Branched/ total SCFA%	Branched chain SCFA	NH3	pH	DMCV
Acetate	Pearson r	1	.781**		.934**							-.782**	
	Sig.		0.008		0							0.008	
Propionate	Pearson r	.781**	1		.925**	-.703*	.934**						
	Sig.	0.008			0	0.023	0						
Butyrate	Pearson r			1	.645*	-.757*		.896**				-.654*	
	Sig.				0.044	0.011		0				0.04	
Total SCFA	Pearson r	.934**	.925**	.645*	1		.737*					-.642*	
	Sig.	0	0	0.044			0.015					0.046	
Acetate/total SCFA%	Pearson r		-.703*	-.757*		1	-.714*					.680*	
	Sig.		0.023	0.011			0.02					0.03	
Propionate/total SCFA%	Pearson r		.934**		.737*	-.714*	1					-.860**	
	Sig.		0		0.015	0.02						0.001	
Butyrate/total SCFA%	Pearson r			.896**				1					
	Sig.			0									
Branched/total SCFA%	Pearson r								1	.890**		.780**	
	Sig.									0.001		0.008	
Branched chain SCFA	Pearson r								.890**	1			
	Sig.								0.001				
NH3	Pearson r										1		
	Sig.												
pH	Pearson r	-.782**		-.654*	-.642*	.680*	-.860**		.780**			1	
	Sig.	0.008		0.04	0.046	0.03	0.001		0.008				
DMCV	Pearson r												1
	Sig.												

Significant correlations (p <0.05) positive are highlighted in green and negative in pink. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Supplementary Table 5-4. Correlations of fermentation products with composition of native *Plantago*.

Monosaccharide composition		DMCV (gas)	Total SCFA	Acetate	Butyrate	Propionate	Ammonia	Branched chain SCFA
Mannose	Pearson r							-.720*
	P-value							0.019
Rhamnose	Pearson r							-.660*
	P-value							0.038
Galacturonic	Pearson r							
	P-value							
Glucose	Pearson r				-.713*	-.647*		
	P-value				0.021	0.043		
Galactose	Pearson r							
	P-value							
Xylose	Pearson r							
	P-value							
Arabinose	Pearson r		.644*	.633*		.643*		
	P-value		0.044	0.049		0.045		
A/X	Pearson r							
	P-value							

		DMCV (gas)	Total SCFA	Acetate	Butyrate	Propionate	Ammonia	Branched chain SCFA
Mucilage	Mucilage yield	Pearson r							
		P-value							
	(CWE/total mucilage)%	Pearson r							
		P-value							
	Water absorption capacity	Pearson r							
		P-value							
	Carbohydrate	Pearson r							
		P-value							
	Protein	Pearson r						.801*	
		P-value						0.03	
	Fat	Pearson r		-.759*	-.797*				
		P-value		0.048	0.032				
Proximate composition	Dietary fibre	Pearson r							-.767*
		P-value							0.044
	Glucose	Pearson r		-.760*		-.849*	-.838*		
		P-value		0.047		0.016	0.019		
	Fructose	Pearson r							
		P-value							
	Sucrose	Pearson r	-.833*	-.773*	-0.591	-.902**	-.765*		
		P-value	0.02	0.042	0.162	0.006	0.045		
	Planteose	Pearson r		.837*	.885**	.768*			
		P-value		0.019	0.008	0.044			
	Raffinose	Pearson r							
		P-value							
Free sugar profile	Stachyose	Pearson r							
		P-value							

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). Significant negative correlations are highlighted in red. Significant positive correlations are highlighted in green.

Supplementary Table 5-5. Correlations of composition of native *Plantago*.

			Galacturonic acid	Glucose	Galactose	Xylose	Arabinose	Mucilage yield%	CWE/total mucilage	Fat	Dietary fibre	Free Glucose	free sucrose	Planteose	Raffinose
	Galacturonic acid	R	1												
		Sig.													
Monosaccharide composition	Glucose	R		1							-.799*	.890**	.825*		
		Sig.									0.031	0.007	0.022		
	Galactose	R			1									.819*	.939**
		Sig.												0.024	0.002
	Xylose	R				1		.965**	.872*		.855*				
		Sig.						0	0.011		0.014				
	Arabinose	R					1	.884**	.960**		.861*				
		Sig.						0.008	0.001		0.013				
	Mucilage yield%	R				.965**	.884**	1	.889**		.859*				
		Sig.				0	0.008		0.008		0.013				
Mucilage yield%	CWE/total mucilage	R				.872*	.960**	.889**	1	-.823*					
		Sig.				0.011	0.001	0.008		0.023					
	Fat	R							-.823*	1				-.874*	
		Sig.							0.023					0.01	
Proximate composition	Dietary fibre	R		-.799*		.855*	.861*	.859*			1				
		Sig.		0.031		0.014	0.013	0.013							
	Free Glucose	R		.890**								1	.817*		
		Sig.		0.007									0.025		
	free sucrose	R		.825*								.817*	1		
		Sig.		0.022								0.025			
Free sugars and oligos	Planteose	R			.819*					-.874*				1	.857*
		Sig.			0.024					0.01					0.014
	Raffinose	R			.939**									.857*	1
		Sig.			0.002									0.014	

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). Correlation is significant at the 0.01 level (2-tailed). Significant negative correlations are highlighted in red. Significant positive correlations are highlighted in green

Supplementary Table 5-6. End products of fermentation of *Plantago* WSF substrates. Values are mean and standard deviation for 0 h, 10 h, 24 h and 48 h.

Level of sub	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
DEW	0	2	0.49760048	0.01682773	0.14804309	0.00493431	0.03973390	0.00649973	0.73194533	0.04568463	68.0440771	1.94795257	20.2443716	0.58942358	5.4668947	1.22922524	0.06244657	0.03766601	0.04656785	0.03042231	1.27573370	0.06241422	6.71000000	0.04242641
DEW	10	2	1.99928165	0.20527764	0.66400565	0.09458691	0.24885175	0.07044323	3.14358368	0.35780763	63.6394098	0.71348604	21.0879321	0.60862645	7.8394327	1.34856096	0.07433225	0.01243701	0.23144463	0.01250015	2.09598625	0.17021575	6.82500000	0.03535534
DEW	24	2	2.64062116	0.27846616	0.82767064	0.12247509	0.40655027	0.05093493	4.13431797	0.46096730	63.8924327	0.38838476	19.9785544	0.73483678	9.8259452	0.13643210	0.06303068	0.00482884	0.25947589	0.00909112	2.04664624	0.02382074	6.87500000	0.07778175
DEW	48	5	3.69126953	0.34821157	1.12856095	0.06852361	0.57119962	0.19900711	5.81311609	0.30175767	63.3485127	4.10415996	19.4120374	0.51098298	9.8624689	3.44366786	0.07276981	0.00423722	0.42208599	0.01051638	2.02635479	0.71022011	6.63400000	0.03911521
LOX	0	2	0.49324974	0.02863746	0.15054700	0.01126853	0.04154763	0.00754781	0.76172654	0.05625624	64.7920469	1.02557991	19.7631875	0.01975831	5.4326281	0.58966214	0.10012138	0.00416159	0.07638217	0.00880245	1.03893636	0.37147600	6.73500000	0.02121320
LOX	10	2	1.96012149	0.13925787	0.55929416	0.04929943	0.20581399	0.01326029	2.91171211	0.22087164	67.3308382	0.32478664	19.1994500	0.23674345	7.0715596	0.08101007	0.06398152	0.00169053	0.18648247	0.01905405	2.10758334	0.15155310	6.80500000	0.02121320
LOX	24	2	2.87863245	0.13212485	0.81560988	0.02956091	0.53124702	0.01523079	4.56047768	0.17841856	63.1129197	0.42801835	17.8853161	0.05152551	11.6513173	0.12185845	0.07350447	0.00254634	0.33498833	0.00150202	2.06784311	0.32328839	6.82000000	0.01414214
LOX	48	5	3.45921591	0.13261464	1.01651008	0.04548603	0.69596208	0.04420424	5.58061178	0.19171996	61.9825478	0.76186691	18.2106523	0.26585549	12.4750656	0.75221564	0.07331734	0.00245562	0.40892371	0.01204026	2.26247654	0.18219120	6.63600000	0.05319774
PBel	0	2	0.59047326	0.00812498	0.17016455	0.00847243	0.09015187	0.00345445	0.93979345	0.00916065	62.8288812	0.25212422	18.1030566	0.72506047	9.5913966	0.27408343	0.09476666	0.01251268	0.08900377	0.01089121	1.19059341	0.31359341	6.70000000	0.02828427
PBel	10	2	2.39136023	0.16120380	0.70271567	0.05927518	0.27163035	0.01575486	3.59737082	0.26318703	66.4892416	0.38325957	19.5261280	0.21918633	7.5550005	0.11477609	0.06429630	0.00278849	0.23166458	0.02695320	1.47636584	0.17552710	6.69000000	0.02828427
PBel	24	2	2.83549133	0.01669617	0.95546092	0.00867555	0.49594937	0.02275310	4.58822130	0.00467274	61.7995758	0.42682982	20.8243189	0.21029108	10.8089391	0.48489431	0.06567166	0.00152227	0.30131968	0.00729136	2.46409342	0.12309310	6.83000000	0.00000000
PBel	48	5	3.55254441	0.14852531	1.35506541	0.04929602	0.64783523	0.04481248	6.01151578	0.23305791	59.0919417	0.51640900	22.5429076	0.07654726	10.7689426	0.42593687	0.07596208	0.00360940	0.45607074	0.01177221	2.37494783	0.71563626	6.60200000	0.02167948
PCun	0	2	0.54442432	0.02872547	0.16082621	0.01152585	0.08776366	0.00165631	0.87282992	0.05165969	62.3864946	0.40136105	18.4190189	0.23035863	10.0670873	0.40607186	0.09127399	0.00577074	0.07981573	0.00975206	0.82938699	0.12901551	6.76000000	0.02828427
PCun	10	2	1.93676433	0.27790907	0.56277384	0.01353180	0.20895836	0.03561223	2.94999987	0.40073477	65.6186042	0.50686446	19.2232892	2.15263071	7.0665406	0.24725932	0.08091566	0.01398507	0.24150334	0.07368167	2.11130933	0.01361031	6.78000000	0.00000000
PCun	24	2	2.76419945	0.11522020	0.80463573	0.02269502	0.55932690	0.04571965	4.47368632	0.20242444	61.7929469	0.22048545	17.9929085	0.30683914	12.4922625	0.45672079	0.07721882	0.00070604	0.34552424	0.01878957	2.11896715	0.07492696	6.79500000	0.04949747

Level of subsp	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
PCun	48	5	3.38864020	0.24016337	1.13956907	0.07853865	0.75828117	0.03280385	5.68073849	0.30213258	59.6076137	1.23256728	20.0481766	0.46986094	13.3852649	1.04208764	0.06958945	0.00535130	0.39424806	0.01757286	2.53362982	0.44479134	6.60000000	0.03082207
PDeb	0	2	0.47584060	0.02037854	0.13677704	0.00743611	0.05554012	0.01864863	0.74840362	0.04961154	63.6302997	1.49510699	18.2830775	0.21838641	7.3547149	2.00424455	0.10731908	0.00290751	0.08024586	0.00314827	1.16353861	0.13250207	6.71500000	0.02121320
PDeb	10	2	2.24775934	0.03450647	0.64020193	0.01156959	0.25852445	0.00648762	3.37775025	0.04976167	66.5457454	0.04121644	18.9530349	0.06330373	7.6531624	0.07932139	0.06848057	0.00183842	0.23126453	0.00280200	1.43834581	0.00442856	6.71000000	0.02828427
PDeb	24	2	2.74406640	0.52487741	0.80314932	0.13272674	0.54776793	0.08161136	4.40770189	0.75417222	62.1471254	1.27461104	18.2307459	0.10809440	12.4513788	0.27890899	0.07170750	0.00887608	0.31271823	0.01495670	1.52617287	0.17093717	6.75500000	0.04949747
PDeb	48	5	3.49973417	0.23782775	1.17219633	0.07417363	0.75178582	0.04661789	5.85262818	0.35628880	59.7797346	0.78700857	20.0266156	0.11980746	12.8457462	0.23223022	0.07347904	0.00937323	0.42891184	0.04949531	2.42046098	0.38654418	6.59200000	0.04324350
PMaj	0	2	0.52396991	0.01030494	0.16406666	0.00556856	0.11447229	0.00185884	0.90610298	0.02534307	57.8334672	0.48028066	18.1053322	0.10816756	12.6355486	0.14826055	0.11425652	0.00520374	0.10359411	0.00761073	0.69110388	0.00511421	6.71000000	0.02828427
PMaj	10	2	2.39276017	0.09685531	0.56657330	0.02384764	0.23907077	0.00591269	3.47382480	0.12552379	68.8742922	0.29943041	16.3328490	1.27666917	6.8834805	0.07852204	0.07909378	0.01055761	0.27542056	0.04660343	1.83437360	0.02244554	6.53000000	0.05656854
PMaj	24	2	2.09634281	0.79381159	0.56099533	0.17851356	0.33426892	0.07361211	3.32091294	1.24736690	63.0865265	0.20750730	17.0887331	1.04325660	10.3816131	1.68281115	0.09443127	0.02518560	0.32930588	0.20142964	1.76615452	0.14887087	6.15500000	0.98287843
PMaj	48	5	3.13300699	0.08333396	0.82556660	0.07369831	0.47517105	0.01786300	4.98892796	0.19090089	62.8327478	1.45817961	16.5219052	0.91417679	9.5438610	0.68284219	0.11101486	0.01059700	0.55518331	0.06888737	2.24115169	0.39786543	6.69600000	0.02880972
PTur	0	2	0.59597545	0.07093995	0.16551132	0.03298604	0.10270857	0.02849438	0.94069439	0.14006521	63.4972782	1.91323021	17.5278293	0.89674761	10.8127284	1.41911258	0.08162164	0.00402630	0.07649905	0.00764483	1.22452169	0.06827584	6.90000000	0.24041631
PTur	10	2	2.07589145	0.10345216	0.61432094	0.05137041	0.21153222	0.03102024	3.16322335	0.23283049	65.6833923	1.56418955	19.4135478	0.19504631	6.6692118	0.48976244	0.08233848	0.00879381	0.26147874	0.04698769	2.59646094	0.11312936	6.79500000	0.00707107
PTur	24	2	2.90649745	0.35279800	0.89779632	0.11976284	0.54622060	0.09094526	4.70312628	0.62273628	61.8447579	0.68745199	19.0880869	0.01901698	11.5875425	0.39942422	0.07479613	0.00269011	0.35261335	0.05923018	2.26671637	0.03887004	6.78000000	0.01414214
PTur	48	5	3.39163747	0.46446249	1.14723300	0.04192212	0.62189596	0.02019140	5.51507981	0.76766142	61.5226919	0.61073669	21.1201473	2.94094802	10.9576961	3.06299570	0.06399465	0.00363852	0.35431338	0.06178688	2.94898741	0.43008591	6.59600000	0.00894427
PVar	0	2	0.66930163	0.25296594	0.18388434	0.07117788	0.04711861	0.02019140	0.99348937	0.38942222	67.5692805	1.02304548	18.5281712	0.09813201	4.7059402	0.18776439	0.09196608	0.00933413	0.09318478	0.04508700	1.21048230	0.14899127	6.73000000	0.02828427
PVar	10	2	2.58773585	0.13741887	0.77830189	0.02801744	0.27169811	0.00800498	3.38018868	0.16143381	64.9988530	0.81626127	19.5562070	0.08926441	6.8359636	0.47838283	0.08608976	0.00248614	0.34245283	0.00400249	2.70283019	0.07071068	6.74500000	0.04949747
PVar	24	2	3.82950738	0.28380621	1.18925759	0.12192516	0.62455045	0.02131530	6.10445570	0.44464603	62.7300756	0.07993210	19.4606793	0.57980641	10.2455212	0.39710350	0.07563724	0.00262635	0.46114028	0.01759936	2.56932281	0.00869585	6.79500000	0.00707107
PVar	48	5	3.91910	0.21043	1.29058	0.04263	0.67743	0.03378	6.35998	0.28515	61.6017	0.60790	20.3018	0.30711	10.6508	0.20792	0.07445	0.00409	0.47285	0.01853	3.11565	0.31882	6.62200	0.04868

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Level of sub	Level of removal	N	AcetDM Mean	AcetDM Std Dev	PropDM Mean	PropDM Std Dev	ButDM Mean	ButDM Std Dev	TotDM Mean	TotDM Std Dev	AcTot Mean	AcTot Std Dev	PrTot Mean	PrTot Std Dev	BuTot Mean	BuTot Std Dev	BrChPpn Mean	BrChPpn Std Dev	BrCh Mean	BrCh Std Dev	NH3DM Mean	NH3DM Std Dev	pH Mean	pH Std Dev
ar			684	667	775	339	424	830	716	103	984	754	849	126	083	283	508	739	833	759	669	053	000	265
W OT	0	2	0.54204449	0.03722145	0.15949665	0.01162106	0.04046581	0.00536275	0.82181115	0.06981942	66.0031102	1.07829324	19.4179533	0.23563120	4.9139942	0.23507017	0.09664942	0.01078854	0.07980420	0.01561415	1.00748082	0.01148409	6.75000000	0.02828427
W OT	10	2	1.99947247	0.13844407	0.38526800	0.03645289	0.24477487	0.05269001	2.83954652	0.14977063	70.3845283	1.16316875	13.6207431	2.00217742	8.5832132	1.40286065	0.07411515	0.00563852	0.21003119	0.00491057	2.01174877	0.06430006	6.80500000	0.02121320
W OT	24	2	2.86265657	0.03343880	0.85586285	0.01418111	0.47525453	0.00819636	4.58732501	0.06293459	62.4044861	0.12720286	18.6567578	0.05318033	10.3599162	0.03654395	0.08578840	0.00037479	0.39355106	0.00711832	1.80418899	0.14195483	6.87000000	0.00000000
W OT	48	5	3.51382367	0.18934391	1.09651893	0.04755213	0.76467612	0.01838703	5.77600588	0.26399064	60.8171102	0.54477220	18.9862284	0.16100122	13.2598136	0.64617659	0.06936848	0.00199600	0.40098716	0.02779352	2.51571247	0.21607239	6.64200000	0.02949576

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Supplementary Table 5-7. End products of fermentation of POV, POV-H and POV-S. Values are mean and standard deviation for 0 h, 10 h, 24 h and 48 h.

Level of Fx	Level of removal	N	AcetDM		PropDM		ButDM		TotDM		AcTot		PrTot		BuTot		BrChPpn		BrCh		NH3DM		pH	
			Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
POV-H	0	2	0.53768562	0.02310929	0.16275631	0.01022342	0.07907270	0.01579173	0.84065742	0.04575519	63.9800917	0.73334506	19.3561731	0.16260729	9.3688112	1.36857297	0.07294924	0.00797835	0.06114279	0.00336925	1.51154330	0.02157018	6.75500000	0.03535534
POV-H	10	2	1.60837435	0.03099209	0.51680777	0.03085548	0.15046332	0.00630623	2.50327760	0.11308754	64.2883750	1.66621750	20.6384622	0.30024450	6.0110961	0.01963708	0.09062067	0.01385610	0.22763216	0.04493374	2.13667207	0.02521754	6.59500000	0.00707107
POV-H	24	2	3.61885430	0.52686660	1.25965594	0.17686444	0.34590468	0.04654071	5.62423994	0.42432099	64.1731407	4.52623817	22.5797966	4.84821520	6.1364963	0.36453394	0.07110566	0.00042557	0.39982503	0.02777812	1.16079052	0.17462861	6.79500000	0.10606602
POV-H	48	5	3.80374599	0.33809532	1.84056848	0.15081761	0.33034869	0.03526094	6.31995511	0.52824549	60.1649032	0.38256697	29.1286206	0.34908703	5.2253218	0.31556959	0.05481154	0.00373934	0.34529194	0.02028835	1.40819424	0.39025844	6.54000000	0.11335784
POV-S	0	2	0.50070021	0.00473116	0.14831432	0.00157705	0.03010892	0.00157705	0.74380187	0.00788527	67.3234966	1.34979472	19.9422745	0.42344008	4.0470796	0.16912171	0.08687149	0.01604113	0.06467842	0.01261643	1.43184647	0.13878071	6.70000000	0.04242641
POV-S	10	2	2.34274995	0.08423636	0.72944014	0.00371369	0.27457197	0.00621543	3.64536879	0.05633918	64.2562903	1.31769644	20.0116533	0.20740585	7.5316585	0.05410040	0.08200398	0.01164391	0.29860674	0.03782631	2.34123254	0.10899803	6.54000000	0.09899495
POV-S	24	2	3.22820427	0.20027299	1.00579800	0.07160943	0.82086128	0.01018849	5.42151896	0.32281567	59.5398460	0.14883338	18.5455116	0.21657413	15.1620819	0.71487508	0.06752560	0.00349468	0.36665541	0.04074477	2.13582806	0.20384106	6.77000000	0.01414214
POV-S	48	5	3.67428145	0.20404937	1.28720036	0.04411376	1.13383456	0.02600352	6.64000801	0.26768687	55.3085073	0.91038950	19.3906995	0.27065870	17.0886810	0.47656211	0.08212112	0.00419002	0.54469164	0.02075165	2.23046963	0.49003690	6.59600000	0.01140175
POV	0	2	0.41339482	0.02255862	0.12451101	0.00268061	0.03857599	0.00523999	0.65693560	0.01717847	62.9043627	1.78900621	18.9544508	0.08760014	5.8845517	0.95151871	0.12256635	0.00749887	0.08045379	0.00282078	1.18516476	0.04069175	6.68000000	0.02828427
POV	10	2	1.98312208	0.00861143	0.60927872	0.01422018	0.21485924	0.00926980	3.03973666	0.00949875	65.2406915	0.48716342	20.0431666	0.40517751	7.0688614	0.32704328	0.07647281	0.00409029	0.23247662	0.01315981	2.20580535	0.19199419	6.63000000	0.01414214
POV	24	2	2.98242935	0.05366373	1.01094743	0.01857792	0.57046762	0.01062020	4.86720534	0.06464083	61.2740947	0.28878262	20.7698905	0.10585313	11.7231228	0.37389273	0.06232892	0.00020743	0.30336095	0.00301939	1.53333650	0.19822554	6.81500000	0.02121320
POV	48	5	3.80602186	0.17051262	1.43535794	0.08055763	0.81148227	0.11799875	6.50490339	0.25295175	58.5068556	1.17646997	22.0793620	1.26816225	12.4628354	1.66246273	0.06950947	0.00356182	0.45204133	0.02739891	1.49823504	0.25871904	6.59800000	0.01788854

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CHAPTER 6

Summary and future directions

Thesis summary

Research presented in this thesis has explored the possibility of identifying *Plantago* variants, both as mutants and as alternative species, that may provide a toolbox for improving or altering the functionality of seed mucilage in food and fibre applications compared to commercial psyllium from *P. ovata*.

P. ovata is a myxospermous species yielding psyllium which is widely used as a dietary fibre due to its high water holding capacity and is used in the food industry as a hydrocolloid in products such as gluten free bread. However, there is minimal natural variation in *P. ovata* which limits its use for creating novel organoleptic properties such as mouthfeel and texture. Also, in spite of its wide use as a dietary fibre supplement, its applications are limited to faecal bulking, as it is very poorly fermented, thereby limiting its production of gastrointestinal health promoting compounds such as short chain fatty acids (SCFA).

In Chapter 3, we investigated a group of *P. ovata* mutants produced by gamma irradiation, to identify mutant lines with differences in mucilage monosaccharide composition, water absorption and mucilage arrangement and studied some of their functional properties which are useful in food and fibre applications. Mutant lines were found to have differences in mucilage architecture such as expansion and layering, the robustness of the mucilage envelope and differences in the amounts and nature of polysaccharides present, as reflected by the monosaccharide composition and the A/X ratios which indicate likely differences in branching and structure. Water absorption and swelling capacity revealed differences in the water affinity and interaction of mutant mucilage with starch. Although stable emulsions were formed mutants did not show improved performance to wild type *P. ovata*. Further investigation of the interactions of starch-mucilage systems through rapid visco analysis showed very subtle differences of mutants in the pasting characteristics as distinct from the wild type *P. ovata*. Although the mucilage from the selected mutants appeared to have differences in composition, these changes were not strong enough to create large enough effects to be exploited in food applications such as gluten free bread, however, the water absorption capacity of mutants would potentially be useful in dietary fibre applications. Previous studies have shown significant differences in properties such as water holding, pasting and

gluten free baking among various species of Plantago. This suggests that the natural variation seems to be greater than induced mutations and possibly more useful for exploitation in food-related uses. It also shows that many different factors rather than mutations in a limited number of genes can influence the technical behaviour of psyllium. *In vitro* fermentations revealed that mutant *P. ovata* only showed very subtle differences in fermentability to the wild type with overall poor fermentability as a consistent feature across all lines. The removal of the cold-water extractable fraction of mucilage reduced the fermentability of the mucilage further, thereby making whole mucilage a better option as a fibre source. The results of food related functionality tests as well as *in vitro* fermentations indicate that the *P. ovata* mutants studied here may not be useful for tailoring food and fibre related functional properties.

In Chapter 4, we described the effect of *P. ovata* whole seed flour or the husk divided into three fractions; cold water (F1), hot water (F2) and residue (F3) on the pasting properties of rice starch and rice flour and their effects when used in gluten free (GF) baking. The alteration of pasting profiles was most prominent for unfractionated husk, WSF, HF3 and WF3. The addition of whole husk and WSF, as well as fractionated husk and WSF, led to changes in the pasting properties. Baking experiments revealed that unfractionated husk and WSF produced high quality GF bread while the addition of other fractions produced poorer qualities in comparison. The F1 and F2 fractions produced better quality than the F3 fractions in GF bread in spite of the minimal changes to pasting profiles, which shows that the majority of the viscosification properties are due to the F3 component of mucilage. F3 had a higher A/X ratio, indicating that the detrimental effects in bread could possibly be due to the self-association of highly branched heteroxylan and phase separation. This demonstrates that *P. ovata* husk or WSF retaining its native mucilage layering ability is important for the production of high-quality GF products, while loss of this natural mucilage arrangement leads to detrimental effects on quality. Therefore, *P. ovata* mucilage fractionation does not appear to be a promising tool for better quality GF products and it is recommended to use the whole mucilage of *P. ovata* for this application.

In Chapter 5, we assessed the *in vitro* fermentability of whole seed flour (WSF) of ten species of *Plantago* including the commercially used *P. ovata* and other Australian native and naturalized species. Although

the fermentation led to the production of gas and SCFA, all species were fermented slowly and poorly and some, like *Plantago major,* were found to be even less fermentable than other species studied. Previous studies have shown that the Australian native and naturalized *Plantago* species possess good water holding capacity with differences in their composition, as well as their effects on starch pasting and GF baking. However, the results of fermentation revealed that in spite of the interspecific variation of the composition of the WSF they are all highly complex in nature making them poorly fermentable. The low fermentability of psyllium from *P. ovata*, in spite of being a viscous soluble fibre, makes it suitable as a bulking fibre supplement for retaining its water-holding capacity throughout the colon. The slowly fermentable species of *Plantago* may also be suitable candidates for fibre applications similar to commercial psyllium. We have also found that planteose, an oligosaccharide first identified in *Plantago* species, is fermentable. Investigation of the differences in the fermentability of the husk, whole seed and de-husked seed of *P. ovata* revealed that the de-husked seed, which is usually a waste product in commercial psyllium production, is more fermentable than the husk and therefore consuming the WSF of *P. ovata* as a fibre supplement, rather than just the husk alone, could reduce wastage, as well as provide better gastrointestinal health effects. In spite of the poor fermentability of Australian native and naturalized *Plantago* species, similar to the commercially used *P. ovata* they are all materials with high water holding capacity. Therefore, we suggest that Australian native and naturalized *Plantago* WSF adapted to the Australian climatic conditions, could possibly be produced commercially as an alternative for imported psyllium from *P. ovata*.

In summary, we have (1) Demonstrated that mutant *P. ovata* may not be suitable as a tool for significantly altering starch pasting and fermentation properties (Chapter 3) (2) Whole mucilage of *P. ovata* is more fermentable than mucilage with the cold water fraction removed (Chapter 3) (3) Native structure of *P. ovata* gives rise to better quality in GF bread than fractionated mucilage (Chapter 4) (4) Demonstrated that Australian native and naturalized *Plantago* species are poorly fermentable (Chapter 5) (5) Shown that the oligosaccharide planteose is fermentable (Chapter 5) (6) Consumption of whole *P. ovata* may provide better gastrointestinal benefits and also reduces wastage compared to consumption of husk only

(Chapter 5) (7) Australian native and naturalized *Plantago* could provide an alternative fibre supplement to commercial psyllium (Chapter 5).

Limitations and future directions

1 – Polysaccharide structural characterization

We recognize that the non-structural nature of the analyses in the work presented in this thesis is a significant limitation. For the mutant *P. ovata* lines, differences were observed in their functionality tests, microscopy and monosaccharide analyses (Chapter 3). However, monosaccharide profiles only show the molar ratios of various monosaccharides and allow an estimation of the degree of substitution using the A/X ratio. Therefore, the true compositional differences in the polysaccharides such as the amount of each type of polysaccharide, length of polysaccharide chains, arrangement of branches on the main polysaccharide chain etc. are not known. Better insights into polysaccharide structure-functionality can be made using the structural characterisation of key polysaccharides in the mucilage but this is technically challenging, particularly for multiple fractions for which gathering sufficient material for analysis is difficult. We, therefore, suggest using methods such as Nuclear Magnetic Resonance (NMR) techniques or Polysaccharide Analysis using Carbohydrate Electrophoresis (PACE) (Mortimer, 2017) for studying the structure as these require smaller amounts of material. However, the utility of PACE in particular is defined by the range of enzymes – xylanases in this case – which are currently available for the digestion of the heteroxylan. The range is limited at the moment as the enzymes are often not able to cleave the backbone due to steric hindrance by the sidechains. The more heavily substituted the backbone, the more reduced the access. Phan *et al.*, (2016) digested the mucilage from several species of *Plantago* using GH10 endoxylanase. This was able to release oligosaccharides from *P. debilis*, *P. lanceolata* and *P. cunninghamii* which have less substituted heteroxylan, however, this method was unable to release oligosaccharides from *P. varia*, *P. major*, *P. ovata* and *P. coronopus* with higher substitution due to steric hindrance.   This method will be more powerful once a bigger selection of xylanases become available – potentially from *Plantago* species themselves.

Although the mutants studied in Chapter 3 did not appear to be promising for use in GF and emulsion systems, the high water absorption capacity may be a useful property in dietary fibre supplements.

Structural characterization of the polysaccharides may help identify the changes from the wild type *P. ovata* which improves the water holding capacity of mutant mucilage.

Similarly in Chapter 4, although we have observed differences in the monosaccharide composition and the behaviour of the fractions of husk and WSF in rice flour and rice starch pasting, the structural differences in the polysaccharides present in each fraction are not known. Discrete temperature fractionation and structural characterization of fractions of husk and WSF could provide an opportunity to better understand their functionality and to confirm the differences in their native structure that leads to their poorer effects in GF baking compared to unfractionated materials.

2 – *In vitro* fermentations of fractionated mucilage of *P ovata*

The *in vitro* fermentation experiment in Chapter 3 was affected by a major state-wide power cut as described in Chapter 1, which led to a loss of the gas production data and possible effects on the fermentation, potentially affecting the quality of the data recovered. However, overall, it is still clear that there were only very subtle differences in the fermentability of the mutants when compared to the WT, so it is not recommended to repeat the same experiment to obtain gas data. However, in the removal of cold-water extractable fractions we have observed a reduction of the fermentability possibly due to the removal of soluble mucilage components such as oligosaccharides, heteroxylan with lower substitution and pectin. Also, the ethanolic extraction performed prior to the cold water extraction removed the simple sugars which further reduced the fermentable carbohydrates present in the substrate used for *in vitro* fermentation. Structural characterization of the mucilage in cold water (CWE), hot water (HWE) and intense agitation extraction (IAE) fractions of *P. ovata* wild type mucilage, followed by *in vitro* fermentation of the whole mucilage, individual fractions and a reconstituted mucilage prepared by mixing individual fractions in the ratios they are naturally found could provide interesting insights on how the solubility, structural differences in the mucilage fractions, as well as how the native arrangement of mucilage in *P. ovata* affects its fermentability. However again the complete structural characterization may not be achieved as methods like PACE for structural analysis of polysaccharides (Mortimer, 2017) may not be

effective due to the difficulty of digesting the heteroxylan using xylanases similar to Phan *et al.*, (2016) who observed greater difficulty in forming oligosaccharides by digestion with the increasing substitution. As shown in the monosaccharides analysis in Chapter 4 the A/X ratios of IAE>HWE>CWE indicating an increasing substitution level with the increased difficulty in mucilage fractionation. Therefore, complete structural characterisation may not be achieved in more substituted fractions like IAE.

Ding *et al.*,(2015) attempted *in vitro* fermentation of fractionated flax seed mucilage. However unlike the sequential mucilage extraction (Cowley *et al.*, 2020) which separates the mucilage into three fractions CWE, HWE and IAE, Ding *et al.*,(2015) used rhamnogalacturonans, arabinoxylans, arabinan-rich rhamnogalacturonans and xyloglucans from flax seed mucilage isolated on separate ion exchange columns, fractionations and dialysis in order to relate structure to fermentability. However, the sequential mucilage extracts prepared here as per Cowley *et al.*, (2020) would include a mixture of different components rather than just a single type of polysaccharide. For example, the CWE may contain simple sugars, oligosaccharides, pectin and less substituted heteroxylan, therefore making understanding of the structure-function relations more difficult.

3 – Safety of *Plantago* whole seed flour consumption

Previous research has indicated that species of Australian native and naturalized *Plantago* are likely to be nutritious but the *in vitro* fermentation work we have carried out has shown that they are poorly fermentable similar to *P. ovata* (Chapter 5) and may be more useful for faecal bulking applications. However, the nutritional, microbiological and toxicological safety of consumption of whole seed flour of *Plantago* needs to be determined as per Australia New Zealand Food Standards Code (Standard 1.1.1 and 1.5.1 – Novel foods). This would involve characterizing anti nutritional compounds, natural toxicants, as well as using animal feeding trials before they can be used as an alternative to commercial psyllium.

4 - Composition of *Plantago* WSF

Out of the ten species of *Plantago* examined here, compositional data is available for seven of them from the previous work of Dr. James Cowley, which has been used for correlation analysis between the composition and the fermentation end products. However, the accessions WOT, LOX and DEW used in this study do not have any compositional data available. Obtaining this data would allow more complete comparisons of composition to fermentability. The compositional data were not available during the period of this project but further research is being undertaken by others at the University of Adelaide to obtain the data to support publication.

The *Plantago* genus has over 400 species (Bala and Gupta, 2011) and it is possible there may be more fermentable species within the genus other than ten species studied in Chapter 5, which are yet to be explored. Hu *et al.*, (2012) conducted a mouse feeding trail with 0.4 g purified polysaccharides extracted from defatted *P. asiatica* seeds per kilogram of body weight and found an increase in the SCFA production, increase in faecal moisture and a reduction in the colonic pH. The control group however was provided with distilled water in a similar quantity making it quite difficult to know how the fermentability of *P. asiatica* compares to substrates of known fermentability like fructooligosaccharides.

Hu *et al.*, (2013) showed that microwave irradiation of *P. asiatica* polysaccharides led to the reduction of average molecular weight, particle size and apparent viscosity with a significant increase in the SCFA production. It is possible that microwave irradiation treatments could enhance the fermentability of the commercially used psyllium from *P. ovata* as well as the other members of the *Plantago* genus including the species studied in Chapter 5.

5 – *In vitro* fermentation of Planteose

Planteose is an oligosaccharide that is present in the endosperm of the *Plantago* species fermented in Chapter 5. Xing *et al.*, (2017) has carried out structural characterization of planteose from another mucilaginous seed, chia. However, there is no literature available on the fermentability of planteose from

any source, although the correlations between the composition of *Plantago* and the end products of fermentation have indicated that planteose is indeed a fermentable oligosaccharide. We suggest the extracting and purifying of planteose and *in vitro* fermentation to determine its fermentability in pure form, coupled to analyses of gas production and fermentation end products. Although the oligosaccharide planteose is found in the *Plantago* species, it is found in the endosperm which is rich in other materials such as mannans, lipids, sugars, and protein (Cowley, O'Donovan and Burton, 2021). However, in *Salvia hispanica*, commonly known as chia, the planteose is found in abundantly in the seed mucilage (Xing *et al.*, 2017). Therefore, chia seeds are recommended as a source for the extraction of planteose. Xing *et al.*, (2017) have shown that solid-phase extraction (SPE) can be used for the extraction of planteose in pure form which can be used as a standard for methods like NMR spectroscopy. Although the SPE method can be used to extract pure planteose it only produces small quantities of planteose, although the starting material was only 40 mg of dry mucilage from chia. As mentioned in Chapters 3 and 5, we used 2 g – 2.5 g substrate in each fermentation bottle so in order to set up an *in vitro* fermentation of planteose, with time removals and sufficient replicates in order to study the gas, SCFA and NH_3 production over time would require large quantities of pure planteose. This would involve numerous extraction cycles which would require resources including time, equipment, labour and capital making it a difficult task. However, if sufficient substrate can be prepared a continued collaboration with the University of Queensland could be pursued to study the fermentability of purified planteose in the future.

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APPENDICES

Appendix 1

Sample details for in vitro fermentation of mutant *P. ovata*

Table 1. Substrates codes for various *P. ovata* mutant substrates

Substrate code	Material	Treatment 1	Treatment 2
M1_W	*P.ovata* WT	Free sugars removed by ethanolic extraction	Whole
M1_F	*P.ovata* WT	Free sugars removed by ethanolic extraction	Cold water fraction removed
M2_W	671-6	Free sugars removed by ethanolic extraction	Whole
M2_F	671-6	Free sugars removed by ethanolic extraction	Cold water fraction removed
M3_W	671-7	Free sugars removed by ethanolic extraction	Whole
M3_F	671-7	Free sugars removed by ethanolic extraction	Cold water fraction removed
M4_W	673-1	Free sugars removed by ethanolic extraction	Whole
M4_F	673-1	Free sugars removed by ethanolic extraction	Cold water fraction removed
M5_W	668-10	Free sugars removed by ethanolic extraction	Whole
M5_F	668-10	Free sugars removed by ethanolic extraction	Cold water fraction removed

Table 2. Substrate weights for each time removal

SubNo	Substrate	Replicate	(h)	Initial weight (g)	Dry matter weight (g)
1	M1_W	A	72	0.2035	0.1765
1	M1_W	B	72	0.2038	0.1767
1	M1_W	C	72	0.2026	0.1757
1	M1_W	D	72	0.2018	0.1750
1	M1_W	E	72	0.2026	0.1757
1	M1_W	A	48	0.2019	0.1751
1	M1_W	B	48	0.2013	0.1746
1	M1_W	A	24	0.2069	0.1794
1	M1_W	B	24	0.2022	0.1753
1	M1_W	A	10	0.2083	0.1806
1	M1_W	B	10	0.2015	0.1747
1	M1_W	A	4	0.2058	0.1785
1	M1_W	B	4	0.2024	0.1755
1	M1_W	A	0	0.2014	0.1746
1	M1_W	B	0	0.2064	0.1790
2	M1_F	A	72	0.2015	0.1976
2	M1_F	B	72	0.2022	0.1983
2	M1_F	C	72	0.2013	0.1974
2	M1_F	D	72	0.2009	0.1970
2	M1_F	E	72	0.2024	0.1985
2	M1_F	A	48	0.2041	0.2001
2	M1_F	B	48	0.2031	0.1991
2	M1_F	A	24	0.2015	0.1976
2	M1_F	B	24	0.2015	0.1976
2	M1_F	A	10	0.2004	0.1965
2	M1_F	B	10	0.2016	0.1977
2	M1_F	A	4	0.2038	0.1998
2	M1_F	B	4	0.2036	0.1996
2	M1_F	A	0	0.2015	0.1976
2	M1_F	B	0	0.2031	0.1991
3	M2_W	A	72	0.2030	0.1777
3	M2_W	B	72	0.2027	0.1774
3	M2_W	C	72	0.2029	0.1776
3	M2_W	D	72	0.2038	0.1784
3	M2_W	E	72	0.2018	0.1766
3	M2_W	A	48	0.2025	0.1772
3	M2_W	B	48	0.2013	0.1762
3	M2_W	A	24	0.2013	0.1762
3	M2_W	B	24	0.2016	0.1764
3	M2_W	A	10	0.2018	0.1766
3	M2_W	B	10	0.2028	0.1775

3	M2_W	A	4	0.2021	0.1769
3	M2_W	B	4	0.2018	0.1766
3	M2_W	A	0	0.2018	0.1766
3	M2_W	B	0	0.2017	0.1765
4	M2_F	A	72	0.2003	0.1839
4	M2_F	B	72	0.2011	0.1847
4	M2_F	C	72	0.2011	0.1847
4	M2_F	D	72	0.2008	0.1844
4	M2_F	E	72	0.2006	0.1842
4	M2_F	A	48	0.2032	0.1866
4	M2_F	B	48	0.2012	0.1848
4	M2_F	A	24	0.2038	0.1871
4	M2_F	B	24	0.2019	0.1854
4	M2_F	A	10	0.2010	0.1846
4	M2_F	B	10	0.2019	0.1854
4	M2_F	A	4	0.2005	0.1841
4	M2_F	B	4	0.2027	0.1861
4	M2_F	A	0	0.2033	0.1867
4	M2_F	B	0	0.2024	0.1859
5	M3_W	A	72	0.2013	0.1749
5	M3_W	B	72	0.2030	0.1764
5	M3_W	C	72	0.2014	0.1750
5	M3_W	D	72	0.2025	0.1760
5	M3_W	E	72	0.2011	0.1748
5	M3_W	A	48	0.2007	0.1744
5	M3_W	B	48	0.2022	0.1757
5	M3_W	A	24	0.2008	0.1745
5	M3_W	B	24	0.2019	0.1755
5	M3_W	A	10	0.2045	0.1777
5	M3_W	B	10	0.2010	0.1747
5	M3_W	A	4	0.2002	0.1740
5	M3_W	B	4	0.2031	0.1765
5	M3_W	A	0	0.2014	0.1750
5	M3_W	B	0	0.2006	0.1743
6	M3_F	A	72	0.2012	0.1874
6	M3_F	B	72	0.2019	0.1881
6	M3_F	C	72	0.2008	0.1870
6	M3_F	D	72	0.2013	0.1875
6	M3_F	E	72	0.2011	0.1873
6	M3_F	A	48	0.2016	0.1878
6	M3_F	B	48	0.2016	0.1878
6	M3_F	A	24	0.2004	0.1867
6	M3_F	B	24	0.2008	0.1870
6	M3_F	A	10	0.2016	0.1878
6	M3_F	B	10	0.2019	0.1881
6	M3_F	A	4	0.2020	0.1882

6	M3_F	B	4	0.2033	0.1894
6	M3_F	A	0	0.2015	0.1877
6	M3_F	B	0	0.2015	0.1877
7	M4_W	A	72	0.2025	0.1743
7	M4_W	B	72	0.2004	0.1724
7	M4_W	C	72	0.2019	0.1737
7	M4_W	D	72	0.2010	0.1730
7	M4_W	E	72	0.2057	0.1770
7	M4_W	A	48	0.2029	0.1746
7	M4_W	B	48	0.2025	0.1743
7	M4_W	A	24	0.2025	0.1743
7	M4_W	B	24	0.2033	0.1749
7	M4_W	A	10	0.2049	0.1763
7	M4_W	B	10	0.2039	0.1755
7	M4_W	A	4	0.2010	0.1730
7	M4_W	B	4	0.2022	0.1740
7	M4_W	A	0	0.2026	0.1743
7	M4_W	B	0	0.2017	0.1736
8	M4_F	A	72	0.2016	0.1953
8	M4_F	B	72	0.2010	0.1947
8	M4_F	C	72	0.2043	0.1979
8	M4_F	D	72	0.2019	0.1956
8	M4_F	E	72	0.2013	0.1950
8	M4_F	A	48	0.2019	0.1956
8	M4_F	B	48	0.2016	0.1953
8	M4_F	A	24	0.2017	0.1954
8	M4_F	B	24	0.2034	0.1971
8	M4_F	A	10	0.2011	0.1948
8	M4_F	B	10	0.2009	0.1946
8	M4_F	A	4	0.2042	0.1978
8	M4_F	B	4	0.2041	0.1977
8	M4_F	A	0	0.2076	0.2011
8	M4_F	B	0	0.2036	0.1973
9	M5_W	A	72	0.2014	0.1748
9	M5_W	B	72	0.2013	0.1747
9	M5_W	C	72	0.2034	0.1765
9	M5_W	D	72	0.2006	0.1741
9	M5_W	E	72	0.2010	0.1744
9	M5_W	A	48	0.2010	0.1744
9	M5_W	B	48	0.2017	0.1750
9	M5_W	A	24	0.2017	0.1750
9	M5_W	B	24	0.2027	0.1759
9	M5_W	A	10	0.2016	0.1750
9	M5_W	B	10	0.2043	0.1773
9	M5_W	A	4	0.2010	0.1744
9	M5_W	B	4	0.2044	0.1774

9	M5_W	A	0	0.2007	0.1742
9	M5_W	B	0	0.2052	0.1781
10	M5_F	A	72	0.2006	0.1893
10	M5_F	B	72	0.2017	0.1903
10	M5_F	C	72	0.2008	0.1895
10	M5_F	D	72	0.2011	0.1897
10	M5_F	E	72	0.2017	0.1903
10	M5_F	A	48	0.2023	0.1909
10	M5_F	B	48	0.2027	0.1913
10	M5_F	A	24	0.2028	0.1914
10	M5_F	B	24	0.2042	0.1927
10	M5_F	A	10	0.2026	0.1912
10	M5_F	B	10	0.2023	0.1909
10	M5_F	A	4	0.2017	0.1903
10	M5_F	B	4	0.2015	0.1901
10	M5_F	A	0	0.2046	0.1931
10	M5_F	B	0	0.2019	0.1905
11	Bl_Fae	A	72	0.0000	0.0000
11	Bl_Fae	B	72	0.0000	0.0000
11	Bl_Fae	A	0	0.0000	0.0000
11	Bl_Fae	B	0	0.0000	0.0000

Appendix 2

Table 1. Substrates codes for various *Plantago* substrates

Substrate code	Material
POV-H	*P.ovata* husk
POV-S	*P.ovata* de-husked seed
POV	P.ovata whole seed flour
Pmaj	*Plantago major*
Pdeb	*Plantago debilis*
Pbel	Platago belidoides
Ptur	*Plantago turrifera*
Pcun	*Plantago cunninghamii*
Pvar	*Plantago varia*
WOT	WOT
DEW	DEW
LOX	LOX
WBR	Wheat bran

Table 2. Substrate weights for each time removal

SubNo	Substrate	Replicate	(h)	Initial weight (g)	Dry matter weight (g)
1	POV-H	A	48	0.2534	0.2340
1	POV-H	B	48	0.2534	0.2340
1	POV-H	C	48	0.2453	0.2265
1	POV-H	D	48	0.2540	0.2345
1	POV-H	E	48	0.2497	0.2306
1	POV-H	A	24	0.2520	0.2327
1	POV-H	B	24	0.2842	0.2624
1	POV-H	A	10	0.2585	0.2387
1	POV-H	B	10	0.2615	0.2415
1	POV-H	A	0	0.2126	0.1963
1	POV-H	B	0	0.2219	0.2049
2	POV-S	A	48	0.2505	0.2316
2	POV-S	B	48	0.2403	0.2222
2	POV-S	C	48	0.2410	0.2228
2	POV-S	D	48	0.2511	0.2322
2	POV-S	E	48	0.2368	0.2189
2	POV-S	A	24	0.2505	0.2316
2	POV-S	B	24	0.2629	0.2431
2	POV-S	A	10	0.2445	0.2260
2	POV-S	B	10	0.2618	0.2420
2	POV-S	A	0	0.2085	0.1928
2	POV-S	B	0	0.2085	0.1928
3	POV	A	48	0.2460	0.2280
3	POV	B	48	0.2553	0.2366
3	POV	C	48	0.2426	0.2249
3	POV	D	48	0.2510	0.2326
3	POV	E	48	0.2509	0.2325
3	POV	A	24	0.2415	0.2238
3	POV	B	24	0.2464	0.2284
3	POV	A	10	0.2495	0.2312
3	POV	B	10	0.2640	0.2447
3	POV	A	0	0.2195	0.2034
3	POV	B	0	0.2129	0.1973
4	Pmaj	A	48	0.2507	0.2317
4	Pmaj	B	48	0.2519	0.2328
4	Pmaj	C	48	0.2530	0.2338
4	Pmaj	D	48	0.2427	0.2243
4	Pmaj	E	48	0.2426	0.2242
4	Pmaj	A	24	0.2440	0.2255
4	Pmaj	B	24	0.2397	0.2215
4	Pmaj	A	10	0.2429	0.2245
4	Pmaj	B	10	0.2456	0.2270
4	Pmaj	A	0	0.2049	0.1894

4	Pmaj	B	0	0.2179	0.2014
5	Pdeb	A	48	0.2427	0.2271
5	Pdeb	B	48	0.2397	0.2243
5	Pdeb	C	48	0.2431	0.2275
5	Pdeb	D	48	0.2442	0.2286
5	Pdeb	E	48	0.2468	0.2310
5	Pdeb	A	24	0.2458	0.2301
5	Pdeb	B	24	0.2494	0.2334
5	Pdeb	A	10	0.2445	0.2288
5	Pdeb	B	10	0.2442	0.2286
5	Pdeb	A	0	0.2061	0.1929
5	Pdeb	B	0	0.2005	0.1877
6	Pbel	A	48	0.2541	0.2378
6	Pbel	B	48	0.2482	0.2323
6	Pbel	C	48	0.2423	0.2267
6	Pbel	D	48	0.2425	0.2269
6	Pbel	E	48	0.2530	0.2368
6	Pbel	A	24	0.2451	0.2294
6	Pbel	B	24	0.2459	0.2301
6	Pbel	A	10	0.2437	0.2281
6	Pbel	B	10	0.2399	0.2245
6	Pbel	A	0	0.2035	0.1904
6	Pbel	B	0	0.2043	0.1912
7	Ptur	A	48	0.2415	0.2235
7	Ptur	B	48	0.2490	0.2305
7	Ptur	C	48	0.2475	0.2291
7	Ptur	D	48	0.2457	0.2274
7	Ptur	E	48	0.2475	0.2291
7	Ptur	A	24	0.2497	0.2311
7	Ptur	B	24	0.2473	0.2289
7	Ptur	A	10	0.2428	0.2247
7	Ptur	B	10	0.2524	0.2336
7	Ptur	A	0	0.2042	0.1890
7	Ptur	B	0	0.2026	0.1875
8	Pcun	A	48	0.2378	0.2216
8	Pcun	B	48	0.2456	0.2289
8	Pcun	C	48	0.2409	0.2245
8	Pcun	D	48	0.2422	0.2257
8	Pcun	E	48	0.2481	0.2312
8	Pcun	A	24	0.2417	0.2252
8	Pcun	B	24	0.2418	0.2253
8	Pcun	A	10	0.2461	0.2293
8	Pcun	B	10	0.2483	0.2314
8	Pcun	A	0	0.2075	0.1934
8	Pcun	B	0	0.2025	0.1887
9	Pvar	A	48	0.2445	0.2274
9	Pvar	B	48	0.2443	0.2272
9	Pvar	C	48	0.2516	0.2340
9	Pvar	D	48	0.2443	0.2272

9	Pvar	E	48	0.2411	0.2242
9	Pvar	A	24	0.2473	0.2300
9	Pvar	B	24	0.2451	0.2279
9	Pvar	A	10	0.2451	0.2279
9	Pvar	B	10	0.2451	0.2279
9	Pvar	A	0	0.2112	0.1964
9	Pvar	B	0	0.2033	0.1891
10	WOT	A	48	0.2320	0.2141
10	WOT	B	48	0.2333	0.2153
10	WOT	C	48	0.2354	0.2173
10	WOT	D	48	0.2378	0.2195
10	WOT	E	48	0.2372	0.2189
10	WOT	A	24	0.2373	0.2190
10	WOT	B	24	0.2302	0.2125
10	WOT	A	10	0.2346	0.2165
10	WOT	B	10	0.2335	0.2155
10	WOT	A	0	0.2000	0.1846
10	WOT	B	0	0.2032	0.1876
11	DEW	A	48	0.2473	0.2299
11	DEW	B	48	0.2572	0.2391
11	DEW	C	48	0.2493	0.2318
11	DEW	D	48	0.2467	0.2293
11	DEW	E	48	0.2424	0.2253
11	DEW	A	24	0.2540	0.2361
11	DEW	B	24	0.2559	0.2379
11	DEW	A	10	0.2493	0.2318
11	DEW	B	10	0.2579	0.2398
11	DEW	A	0	0.2106	0.1958
11	DEW	B	0	0.2400	0.2231
12	LOX	A	48	0.2486	0.2311
12	LOX	B	48	0.2506	0.2322
12	LOX	C	48	0.2421	0.2243
12	LOX	D	48	0.2416	0.2239
12	LOX	E	48	0.2442	0.2263
12	LOX	A	24	0.2487	0.2305
12	LOX	B	24	0.2568	0.2380
12	LOX	A	10	0.2575	0.2386
12	LOX	B	10	0.2437	0.2258
12	LOX	A	0	0.2050	0.1900
12	LOX	B	0	0.2079	0.1926
13	WBR	A	48	0.2645	0.2376
13	WBR	B	48	0.2591	0.2327
14	BL	A	48	0.0000	0.0000
14	BL	B	48	0.0000	0.0000
14	BL	A	0	0.0000	0.0000
14	BL	B	0	0.0000	0.0000

Appendix 3 - 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 completion certificate

THE UNIVERSITY
of ADELAIDE

This is to certify that

Miss Migelhewage Silva

has completed

120 hours

of Career and Research Skills Training

01 Mar 2022

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

Date

adelaide.edu.au CRICOS Provider Number 00123M

seek LIGHT

Appendix 4 – Candidature milestones

Date	Milestone
February 2018	Contacted Prof. Rachel Burton via email
	Skype interview with Prof. Rachel Burton
April 2018	Accepted offer for PhD admission for the University of Adelaide-University of Nottingham PhD program
	Offered and accepted Beacon of Enlightenment PhD scholarship from the University of Adelaide
June 2018	Accepted offer for PhD admission from University of Nottingham, UK
July 2018	Started PhD
September 2018	Completed Integrated Bridging Programme -Research
	Appeared in a promotional video for IBPR (iCaRST) for the University website
	Participated in Postgraduate Research Symposium, Waite Campus
October 2018	Attended University of Nottingham-University of Adelaide Joint PhD conference at University of Nottingham, Malaysia Campus
Dec 2018	Completed the "Core Component of Structured Program
February 2019	Participated in 2019, Australian Institute of Food Science and Technology, Summer School
September 2019	Completed "Major Review of Progress for Doctoral Programs"
	Presented at Postgraduate Research Symposium, Waite Campus
October 2019	Completed annual review of progress
September 2020	Participated in Postgraduate Research Symposium, Waite Campus
October 2020	Completed annual review of progress

December 2020	Withdrew from University of Adelaide-University of Nottingham joint PhD programme, due to COVID travel restrictions
January 2021	Relocated to Brisbane, Queensland
February 2022	Started working with Prof. Mike Gidley's team at the Centre of Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, University of Queensland
April 2021	Attended Centre the Centre of Nutrition and Food Sciences, All-day research meeting at the University of Queensland
August 2021	Participated in the workshop on Indigenous Plant Foods for Nutrition and Health by ARC industrial transformation centre for uniquely Australian foods, CNAFS, QAAFI
October 2021	Completed annual review of progress
November 2021	Participated in CSIRO The Food Structure, Digestion and Health (FSDH) Online Conference by Riddet Institute, New Zealand
April 2022	Completed pre-submission review
September 2022	Submitted thesis to Adelaide graduate Centre