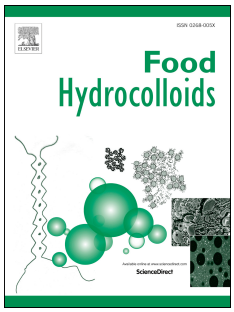

The impact of psyllium gelation behaviour on *in vitro* colonic fermentation properties

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Author contributions

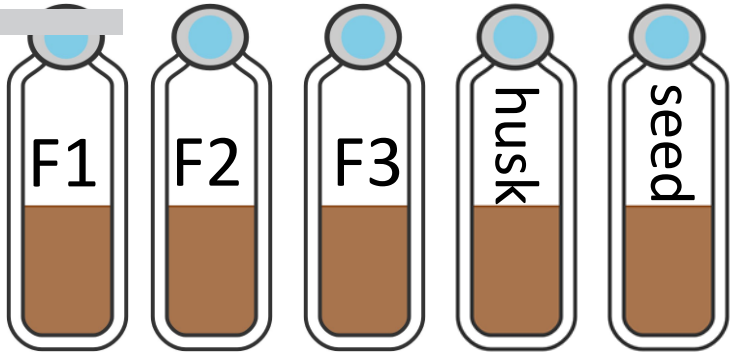
HCH - Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Roles/Writing - original draft
NP – Investigation, Methodology
TK - Formal analysis; Writing - review & editing
YZK - Supervision, Resources
GEY - Conceptualization; Resources; Funding acquisition; Writing - review & editing.
FJW - Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation; Writing - review & editing

Psyllium

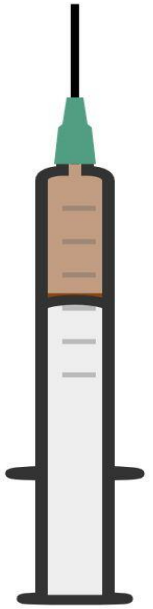
cold water → F1

hot water → F2

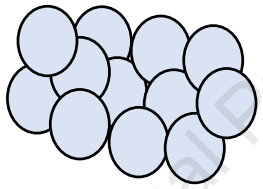
alkali extracted → F3



Sample at 0, 3, 6, 24, 48, 72 h

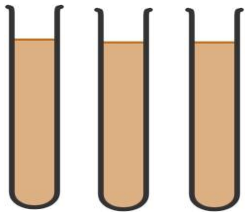


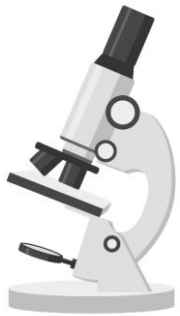
Gas measured

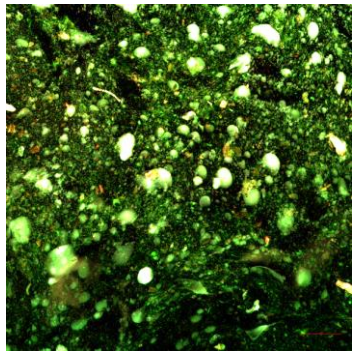


Chemonx image

Metabolomics







FISH

The impact of psyllium gelation behaviour on *in vitro* colonic fermentation properties

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Abstract

Psyllium is a viscous, gel forming fibre with properties that have led it to be used for alleviating gastrointestinal discomfort. We have used previously identified fractions of psyllium with differing flow properties. Fraction 1 (F1) forms a non-gelling solution containing rhamnose. galactose, and arabinose. Fraction 2 (F2) forms a fluid-like gel containing mainly xylose and arabinose, Fraction 3 (F3) has almost identical monosaccharide and linkage composition to F2, but forms an insoluble, self-supporting gel. We performed *in vitro* batch fermentation experiments seeded with human stool. Metabolomics were performed using ^{1}H-NMR, and FISH with calcofluor white and direct red 23 were used to visualise the gels after *in vitro* fermentation of the fractions. The total amount of gas and short chain fatty acid produced was significantly higher for F1, compared to F2 and F3. F3 gas production was significantly lower than F2, but metabolite production between F2 and F3 did not differ. All fractions preferentially lead to the production of propionate instead of butyrate and were produced in the ratio of 58:35:7, 54:38:8, and 61:33:6 (acetate: propionate: butyrate) for F1, F2, and F3 respectively. Microscopy showed differences in how the fractions broke down and demonstrated the localisation of bacteria on the outer edge of each fraction. These results suggest that for these psyllium fractions the structure is a key factor that determines fermentability. Flow properties may play a role in gas production, suggesting directions for future investigation. Isolated fractions may have clinical benefit above that of unrefined psyllium powder aiding in the treatment of gastrointestinal discomfort.

Key words: Psyllium, Hydrogel, Fermentation, Metabolites

1. Introduction

Psyllium, also known as ispaghula or isabgol, is the seed component of the *Plantago ovata* plant commonly found in arid deserts in East Asia and Iran (Dhar, Kaul, Sareen, & Koul, 2005). Psyllium is a dietary fibre consisting primarily of highly branched arabinoxylans and has a propensity to hold water leading to its gelling properties (Yu, Stokes, & Yakubov, 2021; Yu, et al., 2017). These properties of psyllium have resulted in benefits such as improving symptoms of constipation and bloating (Erdogan, et al., 2016; Major, et al., 2018), improving metabolic control such as lowering total and LDL cholesterol (Olson, et al., 1997), reducing plasma glucose and fasting blood glucose (Feinglos, Gibb, Ramsey, Surwit, & McRorie, 2013; Rodríguez-Morán, Guerrero-Romero, & Lazcano-Burciaga, 1998), and increasing feelings of satiety (Brum, Gibb, Peters, & Mattes, 2016).

Psyllium is a viscous (defined here as resistant to flow), gel-forming dietary fibre, which does not undergo digestion within the small intestine. Psyllium reaches the colonic microbiota of the large intestine relatively intact where it is slowly fermented. By some measures of colonic fermentation, Psyllium appears to be non-fermentable within the large intestine (McRorie, 2015). Although direct study of fermentation in the human colon is challenging due to samplingissues, several lines of evidence suggest that Psyllium is fermentable by the human gut microbiota. Marteau, et al., 1994 identified that consumption of psyllium increased propionate and butyrate production in faecal water compared to the placebo. Propionate and butyrate are both produced by fermentation indicating that psyllium is fermented within the human gut (Marteau, et al., 1994). Further clinical studies where individuals consumed psyllium have also observed increases in markers of fermentation such as increased breath hydrogen production, increased faecal short chain fatty concentrations (SCFA) as well as changes in bacterial populations (Gunn, et al., 2020; Jalanka, et al., 2019). Additionally, animal studies have also shown that it is fermented in the cecum and colon (Edwards & Eastwood, 1992). *In vitro* studies have shown that psyllium fermentation results in the production of gas, and SCFA production, albeit at a slower rate than other dietary fibres (Gunn, et al., 2020; Kaur, Rose, Rumpagaporn, Patterson, & Hamaker, 2011). Investigations have indicated that psyllium is a fermentable carbohydrate. Unlike many other dietary carbohydrates, psyllium has been shown to be

comprised of fractions which differ in physicochemical properties and physiological effects (Ren, Yakubov, Linter, MacNaughtan, & Foster, 2020; Yu, et al., 2021; Yu, et al., 2019; Yu, Yakubov, Martínez-Sanz, Gilbert, & Stokes, 2018; Yu, et al., 2017). Marlett, Kajs, and Fischer (2000) identified that psyllium could be divided into three fractions which they termed Fraction A, B, and C. Fraction A was alkali insoluble; Fraction B was alkali soluble and acid insoluble, and Fraction C was alkali and acid soluble. These fractions differed in their structural and physicochemical characteristics with Fraction A consisting mainly of arabinose, but also galactose, glucose, and mannose. Fraction B was mainly comprised of xylose and formed a gel. Fraction C was viscous and primarily composed of xylose, uronic acids, and rhamnose. These differences had an impact on the fermentability of these substrates where it was rapid for fraction C, generating high concentrations of SCFA, whereas Fraction B was poorly fermented leading to low SCFA production when using an *in vitro* fermentation model seeded with rat caecal contents (Marlett & Fischer, 2002). Of note, however, these fractions although all from psyllium differed in a range of both physical and chemical properties which are likely responsible for the differing effects occurring during fermentation.

The main SCFA produced within the colon are acetate, propionate, and butyrate and are most commonly produced in the ratio 60:20:20 (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). Acetate acts as a precursor for butyrate, (Duncan, et al., 2004) and is detectable in systemic blood (Bloemen, et al., 2009). Propionate is absorbed, reaching the liver (Bloemen, et al., 2009), where it may play a role in hepatic gluconeogenesis (den Besten, et al., 2013). Propionate has also been demonstrated to be involved in metabolic control by reducing energy intake, increasing satiety hormones such as PYY and GLP-1, reducing total cholesterol (Chambers, et al., 2015), and improving b-cell function and insulin secretion (Pingitore, et al., 2017). Butyrate is used as an energy source for the colonic enterocytes (Roediger, 1980). In addition, these SCFA confer health benefits as ligands for free fatty acid receptors 2 and 3 which have also been associated with a plethora of health benefits and have been reviewed elsewhere (Byrne, Chambers, Morrison, & Frost, 2015; Carretta, Quiroga, López, Hidalgo, & Burgos, 2021).

These metabolites are produced via different pathways, requiring different enzymes from bacteria which utilise the different fibre sources (Duncan, et al., 2004; Reichardt, et al., 2014). Therefore, it is postulated that the colonic microbiota can be manipulated into producing propionate or butyrate by dietary means (Reichardt, et al., 2018). This has been demonstrated with starch, which results in increased butyrate production (Teichmann & Cockburn, 2021). This is less clear for propionate, although rhamnose has been shown to be potentially propiogenic (Gietl, et al., 2012) .

It is difficult to evaluate the impact of dietary fibres on the gut microbiota and their metabolites in humans, therefore *in vitro* models are often employed. These models are also beneficial as they allow more mechanistic analysis of the colonic environment to be performed. Different structural factors are likely to affect SCFA production including sugar linkage (Harris, Edwards, & Morrison, 2017), and composition and branching (Hernot, et al., 2009; Mortensen, Holtug, & Rasmussen, 1988; van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007). These factors also effect the physical properties of the fibre such as substrate solubility, viscosity, and gelation, altering the fibres fermentation properties. The ideal substrate characteristics to produce propionate and butyrate are unclear, although it is likely to be a combination of these properties.

In this paper the term “viscosity” is used as a definition of material’s resistance to flow. It is important to make distinctions between shear viscosity, which is defined as shear stress divided by the shear rate and complex rheological properties, which include the dependency of shear viscosity on shear stress/shear rate (i.e. shear thinning or shear thickeneing behaviour), as well as other flow properties such as extensional viscosity, viscoelasticity, yeld stress and thixotropy. Each fibre and their form under physiological conditions inside the gut is characterised by a complex set of rheological properties. Therefore defining fibre materials as viscous or non-viscous can be erroneous, and one needs to be very careful when describing complex rheology of fibre. For example, human saliva is described as viscous. However, its shear viscosity is not too dissimilar to that of water. The perceived “viscosity” of saliva is associated with high extensional viscosity and viscoelastic effects that lead to the formation of stable liquid bridges (strings) when stretched. Often, “viscosity” can be quantified using such apparatuses as a viscometer or rapid viscosity analyser. These methods and the associated

measures of viscosity may be useful for ranking different materials in accordance with their effective resistance to flow under conditions of the test. Few exceptions aside, however, they fall short in describing the fundamental phsycial parameters that characterise and govern the flow behaviour of fluids, in particular, when such flow behaviour is complex. The three fractions used in this study have the distinct sets of rheological properties. From the rheological standpoint, they represent three different classes of material behaviour and are not sitting on a continuum between viscous fluid and a soft gel. These aspects have been extensively characterised in (Yu, et al., 2021). Approaching this problem with caution and for the purposes of simplicity, we will use the term "viscosity" in order to indicate a relative measure of material's resistance to flow.

In previous work we have demonstrated that with different treatments, psyllium can be separated into distinct fractions, cold water (F1), hot water (F2) and alkali extracted (F3). These fractions were found to have similar monosaccharide composition and sugar linkages but differing physicochemical properties (Yu, et al., 2017). F1 differs the most compared to the other fractions. F1 has 15.1% rhamnose, 9.7% galacturonic acid, and the corresponding sugar linkages are not present in F2 or F3 . Additionally, F1 has lower molar percentage (mol%) of arabinose and xylose compared to F2 and F3. A solution of F1 forms a viscoelastic liquid at 37°C. In contrast, F2 and F3 are similar in structural components but differ vastly in their rheological properties. F2 and F3 have near identical arabinose, xylose, and galactose mol % differing by 2 %, 2.7 %, and 0 % respectively, and the corresponding sugar linkages did not differ. Although F2 and F3 are the same chemically they differ significantly in their rheological properties. The F2 fraction has an intrinsic viscosity of 5.6 dL/g, and F3 has an intrinsic viscosity of 7.4 dL/g. A solution of F2 is considered to be a weak gel at 37°C, whereas under the same conditions F3 forms a much stronger gel network (Table 1) (Yu, et al., 2017). The physicochemical characteristics of these fractions are now known, however it is unknown if these fractions have differing effects on colonic health. Therefore, we used these fractions to determine if the differing gelling profiles impacted the *in vitro* fermentation outcomes. This could then provide further opportunities for development of methods to improve colonic health without unwanted side effects.

Table 1: Psyllium fractions and their characteristics. Information obtained from (Yu, et al., 2017)[1]

	CW F1	HW F2	KOH F3
Seed dry mass	4.50%	3.20%	9.20%
MW	1085	978	953
Ax:Xy	0.2	0.3	0.33
Ramnose Mol%	15.1	ND	ND
Arabinose Mol%	12.3	22.2	24.2
Xylose Mol%	58.2	73.7	71
Galacuronic acid Mol%	9.7	0.4	0.4
Intrinsic viscosity dL/g	3.1	5.6	7.4
Radius of gyration Rg.nm	40	51	53
Viscosity at 37°C	viscoelastic fluid	viscous / gel like	gel like

[1] The seeds used by (Yu, et al., 2017) are from the same batch of seeds used to produce the fractions in this study. Monosaccharide composition was determined using high-performance anion exchange chromatography coupled with pulsed amperometric detection, glycosidic linkage was measured by GC-MS. Rheological properties and radius of gyration were measured by small amplitude oscillatory shear rheometery. Further details can be obtained in (Yu, et al., 2017) where this analysis was performed.

2. Methods

2.1 Substrates

Psyllium (Plantago ovata) seeds were gifted from Professor Rachel Burton (University of Adelaide, Australia) with growth conditions developed at the University of Adelaide and outlined in (Phan, et al., 2020). Fractions of psyllium were extracted from the same batch of seeds as described and characterised in Yu, et al. (2017). The fractions tested were extracted in cold water (F1), hot water (F2), or potassium hydroxide (F3). In addition to the Psyllium fractions, the Psyllium seed and husk were fermented along with glucose (highly fermentable control) and a blank (negative control).

2.2.1 In vitro colon models

In vitro colon models were performed as described in Williams, Bosch, Boer, Verstegen, and Tamminga (2005). Briefly, to a 100ml serum bottle; 76 ml basal media, and 5 ml vitamin buffer solution, was added.

Basal media consisted of 0.7134 g/L KCl, 0.7134 g/L NaCl, 0.2378 CaCl2·2H2O, 0.5945 g/L MgSO4·7H2O, 1.567 g/L PIPES buffer, 0.642 g/L NH4Cl, 1.189 g/L trypticase, 1.174 mL/L resazurin (1g/L), 11.891 mL/L trace mineral solution (25 mg/L $MnCl_2 \cdot 4H_2O$, 20 mg/L $FeSO_4 \cdot 7H_2O$, 25 mg/L $ZnCl_2$, 25 mg/L $CuCl \cdot 2H_2O$, 50 mg/L $CoCl_2 \cdot 6H_2O$, 50 mg/L SeO_2, 250 mg/L $NiCl_2 \cdot 6H_2O$, 250 mg/L $Na_2MoO_4 \cdot 2H_2O$, 31.4 mg/L $NaVO_3$, and 250 mg/L H_3BO_3 to 0.02M HCl), 11.891 mL/L haemin solution (50 mg of haemin to 25 mL of 0.05 M NaOH), and 11.891 L/L fatty acid solution (0.685 mL of acetic acid, 0.3 mL propionic acid, 0.184 mL butyric acid, 0.047 mL isobutyric acid, 0.055 mL 2-methylbutyric acid, 0.055 mL valeric acid and 0.055 mL isovaleric acid added to 100 mL of 0.2M NaOH). The basal media solution was pH corrected to pH 6.8 with concentrated KOH and bubbled overnight with CO_2. To each serum bottle 76 mL of the basal media was dispensed under a constant stream of CO_2.

Vitamin buffer solution was produced by adding 15 mL of vitamin/phosphate solution (27.35 g KH_2PO_4, 10.2 mg biotin, 10.2 mg para-amino benzoic acid, 10.3 mg folic acid 10.3 mg cyanocobalamin, 82 mg calcium *d*-pantothenate, 82 mg nicotinamide, 82 mg riboflavin, 82 mg thiamine HCl and 82 mg pyridoxine HCl to 0.5 L dH_2O) to 60 ml of pre-reduced, sterile 0.77 M Na_2CO_3.

To each prepared serum bottle, 0.5 ± 0.02 g of substrate was added, and performed in duplicate. Due to complex gel formation observed in the psyllium fractions, all test substrates (and the blank) were hydrated for 3 hours under shaking at 80 rpm at 37°C. Once hydrated, all samples were homogenised using an ultra-turrax (IKA T 10 Basic S1, 5mm dispersion diameter) for 3 minutes, under a constant stream of CO_2.

To each serum bottle 1 mL reducing agent (1.0 g L-cysteine HCl solution and 1.0 g Na_2S $9H_2O$ into 50 mL dH_2O and adjusted to pH 10 using concentrated NaOH), was added. Bottles were purged with

CO_2 for a further 3 minutes, sealed airtight, and incubated overnight at 37°C prior to inoculation with a faecal slurry.

2.2.2 Faecal slurry preparation

Faecal samples were collected from 3 healthy individuals. Ethical approval for the study was granted by the Human Research Governance Committee at the Quadram Institute (IFR01/2015) and the London - Westminster Research Ethics Committee (15/LO/2169). Faecal slurries were prepared on the day of collection by mixing stool and pre-reduced PBS, pH 7.4 (P4417, Merck, Darmstadt, Germany) in a 1:10 ratio. The mix was homogenised in a stomacher for 30 seconds at 230 rpm and filtered through a strainer bag (BA6141/STR, Steward limited, UK). Each serum bottle was inoculated by injection of 3.0 mL of faecal slurry and incubated at 37°C for 72 h.

2.2.3 Sampling procedure

Serum bottles were sampled after 0, 3, 6, 24, 48 and 72 hours of fermentation. To maintain anaerobic conditions each bottle was sampled through a butyl rubber stopper with a 19G needle and syringe.

Gas production was measured by measuring the volume of syringe displacement in a 10 mL syringe attached to a 19G needle after inserting into the septum of the serum bottle. If the displacement was above 10 mL the syringe and needle was removed, gas expelled and then re-inserted into the serum bottle for further displacement measurements.

Per serum bottle a 4 mL aliquot of the fermentation liquid was obtained by needle extraction and 2mL aliquoted into a microcentrifuge tube. The tube was centrifuged at 16000 *g* and 4°C for 10 minutes, the supernatant was removed and stored at -20°C for metabolomic analysis by NMR. The pellet was resuspended with 375 µL PBS and 1125 µL of 4% paraformaldehyde (PFA) and incubated for 2 hours at room temperature. The PFA treated pellet was washed twice with 1 mL of PBS and resuspended with 600 µL PBS:100% EtOH (1:1), and stored at -20°C until further analysis by Fluorescence *In Situ* Hybridisation (FISH) microscopy.

2.3 Metabolomics

The samples containing the supernatant from the fermentation media were centrifuged (3,000 x g, 3 min) and 400-µL aliquots were pipetted directly into NMR tubes (Norell® Standard Series™, 5 mm), followed by the addition of 200 µL of phosphate buffer (NaH_2PO_4 [21.7 mM], K_2HPO_4 [82.7 mM], NaN_3 [8.6 mM], 3-(trimethylsilyl)-propionate-d_4 [TMSP, 1.0 mM], prepared in D_2O) (Le Gall, et al., 2019). The spectra were recorded on a Bruker Avance II 500 MHz spectrometer, equipped with an inverse triple resonance z-gradient probe, at a ^1H frequency of 500 MHz. All experiments were acquired at room temperature, using Bruker's '*noesygppr1d*' pulse sequence, with a minimum of 256 scans, π/2 *rf* pulse of 11.57 s, mixing time of 0.1 s, acquisition time of 4.1 s, relaxation delay of 5 s, featuring selective pre-saturation (1.0 ms) on the residual H_2O peak frequency during relaxation delay and mixing time for effective solvent suppression. Spectra were apodised using 0.1 line broadening and referenced using the TMSP peak (0.0 ppm).

2.4 Fluorescence in situ hybridization

The method was adapted from (Gorham, Williams, Gidley, & Mikkelsen, 2016) and (Koev, Harris, Kiamehr, Khimyak, & Warren, 2022). Due to the *viscous* nature of the psyllium samples were embedded for sectioning using a cryotome. Fixed samples were mounted into OCT embedding matrix (CarlRoth, Karlsruhe, Germany). Mounts were then frozen using a dry ice – ethanol bath and stored in dry ice or at -80^{o}C prior to sectioning on the cryotome. For the non-gelatinous substrates an even layer of supernatant was embedded in the OCT. Samples were cryosectioned at -40 ^{o}C and 70 µm slices were taken. Each slice was mounted on a microscope slide and dehydrated by immersing for 3 minutes sequentially in 50%, 80%, and 100% ethanol, and then air dried. For liquid samples 5 uL of fixed sample was pipetted onto a slide and air dried.

Once air dried to each sample 12 µL of hybridisation buffer solution (per 20 ml 3600 µL 5M NaCl, 400 µL 1M Tris-HCl (pH 8.0), 600 µL formamide, 9980 µL double-distilled water (ddH2O) and 20 µL 10% SDS) was added to the sample followed by 5 µL of each probe (Table 2, 50 ng/µL) (Table 1), and incubated in the dark for 1 hour at 50 °C, with humidity created with a tissue soaked with hybridisation buffer. The sample was then washed with wash buffer solution (per ml 12.8 µL 5M NaCl, 20 µL 1M Tris-HCl [pH 8.0], 10 µL 0.5M EDTA [pH 8.0], 96.2 µL ddH2O and 1 µL 10% SDS) and incubated

for 20 min at 50 °C. After the washing, the slide was gently immersed in ice cold water for 3 seconds and air dried.

For counterstaining the arabinoxylan, 5 µL of 0.1 % calcofluor white stain (CFW) was added to each sample and incubated for 30 seconds at room temperature. Slides were then washed with a drop of PBS, and then 5 µL of 0.1% direct red 23 for 5 minutes at room temp. Slides were washed with a drop of PBS and a coverslip was placed on the slide.

2.5 Microscopic evaluation

Slides were imaged using a Zeiss LSM880 confocal microscope using a x 10 objective. Detection wavelengths were as follows; Cy 5: 644-759, Texas Red: 597-651, Cy3: 535-678, ATTO740: 718-758, Calcofluor white: 410-524, Direct Red 23: 528-644. Within the images these probes are shown by the colours, green, cyan, orange, yellow, white, and red respectively and what they excite is depicted in Table 2.

Table 2: The domain and order of specific published oligonucleotide probes used in this study for FISH analysis.

Name	Probe sequence (5'-3')	5' conjugated fluorophore	Target organisms	Reference
Eub338 I	GCTGCCTCCCG TAGGAGT	Cy5	Virtually all *Bacteria*, Kingdom *Eubacteria* (Most but not all bacteria)	(Amann, et al., 1990)
Eub338II	GCAGCCACCC GTAGGTGT	Cy5	*Planctomycetes*	(Daims, Brühl, Amann, Schleifer, & Wagner, 1999)
Eub338III	GCTGCCACCC GTAGGTGT	Cy5	*Verrucomicrobiales*	(Daims, et al., 1999)

Bac303	CCAATGTGGG GGACCTT	TxRed	Bacteroidaceae, Prevotellaceae, some Porphyromonadaceae	(Manz, Amann, Ludwig, Vancanneyt, & Schleifer, 1996)
Bif164	CATCCGGCATT ACCACCC	ATTO740	Bifidobacterium spp.	(Langendijk, et al., 1995)
Lab158	GGTATTAGCA YCTGTTTCCA	Cy3	Lactobacilli, Enterococci	(Hermie J. M. Harmsen, 1999)
Calcofluor white		Binds to β1,3 and β1,4 linked polysaccharides		(Yu, et al., 2017)
Direct red 23		Preferentially binds to β1,4 linked polysaccharides		(Yu, et al., 2017)

2.6 Data analysis

All experiments were conducted with three different stool donors in duplicate. NMR data was processed using NMR Suite v7.6 Profiler (Chenomx®, Edmonton, Canada). Statistical analysis was performed using IBM SPSS Statistics software version 22. Differences between substrates were assessed using ANOVA with Bonferroni correction where applicable. Additionally, unpaired t-tests were performed to see if there was any significant differences specifically between F2 and F3, which have near identical composition but different viscosity. Total SCFA production is calculated as the sum of acetate, propionate, and butyrate. Graphs were produced using Graphpad version 5.04 and data presented are mean with standard deviation. Images were processed in Zeiss Zen Blue (edition version 3.4).

3.0 Results and Discussion

All the psyllium fractions produced measurable gas during *in vitro* fermentation. Detectable gas production was observed at 24 hours for F1, Seed and Husk, and after 48 hours of fermentation for F2 and F3 (Figure 1a). The highest total gas produced after 24 hours was observed for microbial

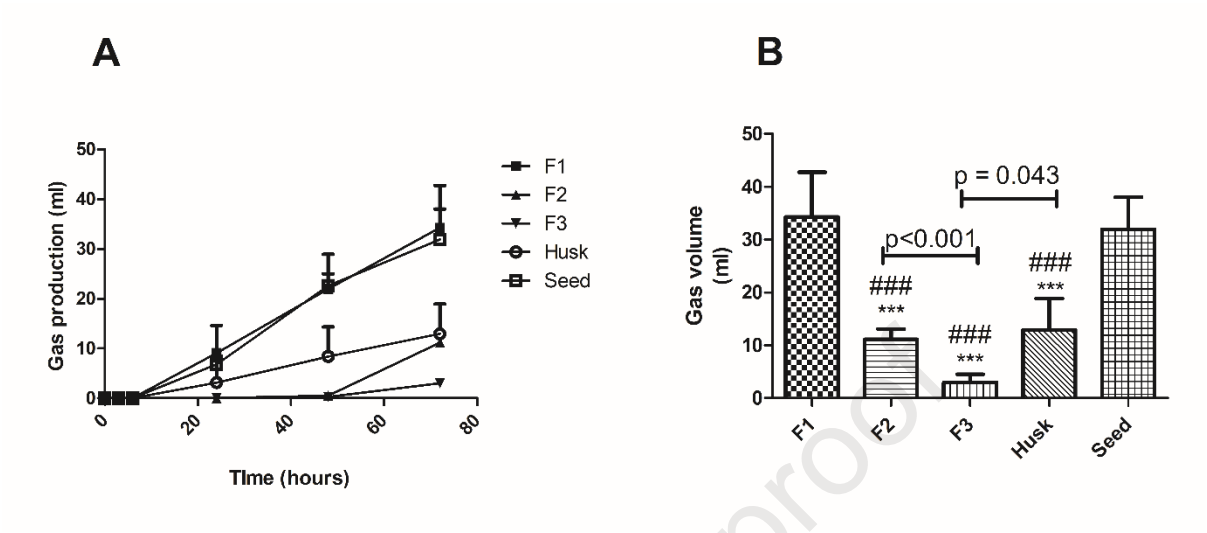
fermentation of seed (31.93 (6.12) mL) and F1 (34.27 (8.46) mL) which was significantly higher than the other fractions tested (figure 1b, $p < 0.0001$). F3 fermentation resulted in the lowest volume of gas production (3.00 (1.50) ml), which was significantly lower than the gas produced from husk fermentation (12.93 (5.94) ml, $p = 0.043$).

Fraction F3 (KOH extraction) was the most viscous of the fractions tested and produced the least gas, which was significantly lower than F2 ($p < 0.001$) and the husk ($p < 0.05$). F2 and F3 have the same structure and linkage, but F3 is more viscous (Table 1, (Yu, et al., 2017)) therefore suggesting that viscosity is an important factor in gas production, in addition to sugar composition.

Gas production from the seed was significanly higher than the husk, F2 and F3. Seeds themselves are not viscous, but once in water they can release mucilage (Yu, et al., 2017). Thus any soluble fermetable mucilage produced from the seed, would be similar to F1 (cold water extraction), as the seed was not exposed to hot water or KOH. In addition within our methodology we utilised the ultraturrex to mix the test materials with our media, likely resulting in the breakdown of the seed structure. Both of these factors likely resulted in the similar gas producing profile observed for the seed and F1.

Highly viscous fibres (including psyllium husk) have been suggested to result in lower gas production when compared to other non-viscous fermentable carbohydrates in the literature. For example *in vitro* psyllium fermentation produced less gas than gum arabic, and carboxymethylcellulose (Bliss, Weimer, Jung, & Savik, 2013), corn arabinoxylan, and β-glucan (Kaur, et al., 2011), however these findings could not be ascribed only to viscosity due to differences in sugar composition and linkage. In the present study F1 was rapidly fermented, generating the most gas of all the substrates tested. This may be due to the lower viscosity observed for this fraction, the pectin within the fraction, or a combination of both. Pectin has been shown to be rapidly fermented leading to the production of gas (Jeraci & Horvath, 1989). The high gas production associated with F1 is also of note as it governs the gas production in the husk samples that contains all three fraction. In the case of the husk material, the amount of gas produced by the F1 (27% of the husk) is offset by low gas producing F3 (54% of the husk), and thefore the gas produced is directly proportional to the amount of each fraction. These data

298 suggest that the viscosity is an important factor in determining detectable gas production, and that F1
299 may be the main gas producing portion of the psyllium husk.



300

301 Figure 1: Gas production by in vitro fermentation. ### indicates $p < 0.0001$ compared to F1 *** $p <$
302 0.0001 compared to seed. N = 3, analysis is ANOVA with post hoc Bonferroni test. Presented is mean
303 + SD. $p < 0.001$ is from unpaired t-test between F2 and F3 only.

304

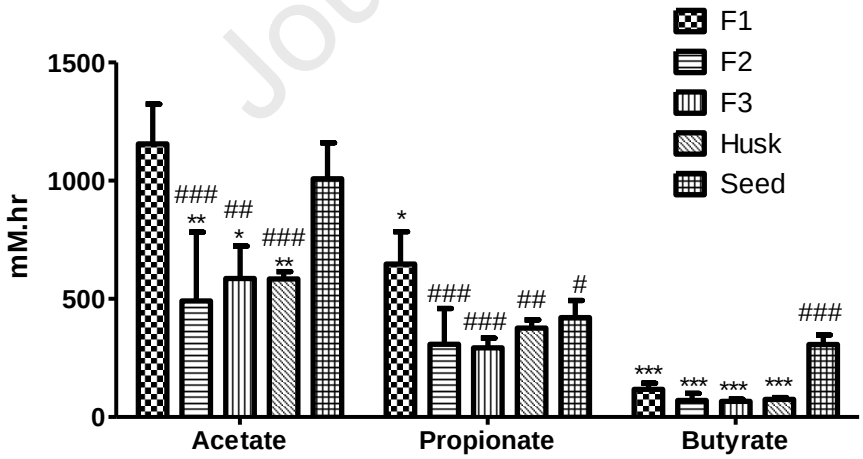
305 **3.2 *In vitro* metabolite production**

306 *In vitro* fermentation resulted in the production of measurable SCFA from all psyllium fractions
307 (Figure 2). Acetate production was the most prominent type of SCFA for all the substrates tested. F1
308 fermentation yielded the highest total SCFA production (1920.60 (313.01) mM.hr after 72 hours
309 which was significantly higher than the other psyllium fractions tested (except seed, $p < 0.01$). F1 and
310 seed fermentation both generated significantly higher acetate production compared to F2, F3 and husk
311 ($p < 0.05$). Propionate production was the highest for F1 (647.32 (136.72) mM.hr), this was
312 significantly higher than all substrates tested (including the seed, $p < 0.05$). Fermentation of the seed
313 substrate resulted in the highest production of butyrate (307.31 (39.83) mM.hr), which was
314 significantly higher than all the other substrates tested ($p < 0.001$). Along with the main SCFAs a
315 variety of other metabolites were measured (Supplementary File 1). Lactate was identified as a
316 fermentation metabolite only in the seed and glucose fermentation vessels, however by 72 hours it

317 was no longer measurable in any of the substrates. Less than 0.1 mM of lactate was observed in any of
318 the samples. Although concentrations were low (< 2.0 mM) each of the psyllium fractions showed
319 increasing concentrations of valerate. This increased valerate concentration may be as a result of the
320 increased production of propionate which is a precursor for valerate (Oliphant & Allen-Vercoe, 2019).

321 F2 and F3 showed very similar SCFA production profiles, despite very different viscosity, suggesting
322 that viscosity was not a key factor in determining SCFA production. F1 has a different structure to F2
323 and F3 where it contains proportionally less arabinose and xylose, but more rhamnose and galactose
324 (Table 1). The increase in SCFA production compared to F2 and F3 indicated that a combination of
325 the chemical composition, sugar linkage and structural motif may be important in determining SCFA
326 production from this substrate. This finding can be compared to the observations of Marlett et al
327 (2002) who also show differences in SCFA production between fractions of psyllium. The fractions of
328 Marlett et al., however, were obtained through different isolation methodologies, which also used acid
329 extraction, and led to psyllium fractions differing in composition, so are not directly comparable to the
330 findings of this study (Marlett & Fischer, 1999; Marlett, et al., 2002; Marlett, et al., 2000).

331



332

333 Figure 2. Area under the curve of SCFA production by bacterial fermentation after 72 hours of
334 fermentation # denotes vs F1 and * denotes vs seed. *, # $p < 0.05$, **,## $p < 0.01$, ***,### $p < 0.001$
335 (ANOVA with post hoc Bonferroni, presented is mean + SD). No differences in F2 and F3 were
336 identified by t-test.
337

3.2.2 SCFA proportion

Table 3: Proportion and total SCFA production after 72 hours of fermentation.

	Acetate %	Propionate %	Butyrate %	Total mM
F1	58.24 (2.22)	35.09 (3.57)[a]	6.66 (2.14)[a]	41.39 (4.35)[a]
F2	53.65 (3.08)	38.46 (2.65)[a]	7.89 (0.67)[a]	28.91 (4.70)[b]
F3	60.71 (4.10)	32.92 (3.80)[a]	6.37 (0.41)[a]	26.72 (5.72)[b]
Husk	55.71 (1.43)	36.78 (1.50)[a]	7.51 (0.49)[a]	28.24 (1.51)[b]
Seed	55.83 (4.82)	24.87 (2.42)[b]	19.30 (3.10)[b]	34.41 (3.55)[ab]

Different letters indicate significant differences based on ANOVA with post hoc Bonferroni correction. Different letters within columns denote statistical differences, presented mean (sd).

Proportionally for all the psyllium fractions tested acetate was the main SCFA produced, then propionate, then butyrate (Table 3). There were no significant differences observed in the molar proportion of acetate produced. The proportion of propionate was over 30 % for all fractions tested, except for seed (24.87 [2.42] %) which was significantly lower than the other fractions ($p < 0.001$). In contrast, seed fermentation generated the highest proportion of butyrate with 19.30 (3.10) %. This was significantly higher than all other substrates ($p < 0.01$), none of which led to greater than 10 % molar ratio of butyrate. No differences in SCFA proportion were identified for F1, F2, or F3, although F1 fermentation did result in the highest yield of SCFA (41.39 [4.35] mM).

The increase in butyrate with the fermentation of seed could be caused by a number of factors related to compositional differences of the seed to the other fractions tested. Cowley et al (2021) identified that *Plantago ovata* (psyllium) seeds were comprised of 51.41 % fibre. When comparing the monosaccharide composition of the seed and husk differences can be observed. When compared to the husk the seed has increased glucose and mannose but decreased xylose (supplementary table 1) (Cowley, et al., 2021; Guo, Cui, Wang, & Young, 2008). Glucose and mannose individually have been demonstrated to be fermentable and produce SCFA (Gietl, et al., 2012). Glucomannans in konjac glucomannan have also shown to produce high amounts of butyrate after *in vitro* fermentation (Bai, et

al., 2021). Furthermore, the seed consists of 7.08 % fat, and as the whole seed underwent homogenisation with the ultraturrex before fermentation, fat would also potentially be fermented (Cowley, et al., 2021). Fermentation of fats could have contributed to SCFA production. For example, Thum et al., (2020) identified that fermentation of milk fat resulted in the increase in SCFA production, and for bovine and ovine milk significanly more butyrate was produced (Thum, Young, Montoya, Roy, & McNabb, 2020). Therefore it is likely that these factors together resulted in the SCFA production and specifically the increased butyrate production compared to the other substrates tested.

All three psyllium fractions (and husk) produced SCFA in an approximate ratio of 57:36:7, indicating that the bacterial fermentation of these substrates favour the production of propionate compared to butyrate. An increased preference of propionate from psyllium has been previously identified in other *in vitro* studies where after 48 hours of fermentation the ratio was 51:35:14 (Kaur, et al., 2011). Interestingly, in comparison to the gas production there were no differences in the proportion of SCFA production between F1, F2, and F3. This suggests that the lower viscosity of F1 compared to F2 and F3 may enable the bacterial enzymes to act on more of the carbohydrate source, as indicated by increased gas and SCFA production compared to F2 and F3. The lack of difference in the molar proportions of the SCFA, or the metabolic intermediates (supplementary file 1) indicate that the bacterial pathways and enzymes do not differ, but the overall production of metabolites is limited by the viscosity of F2 and F3. This however does not account for all of the SCFA production as F1 is the only fraction containing rhamnose which has been previously shown to increase the production of propionate *in vitro* (Gietl, et al., 2012). F2 and F3 do have a higher proportion of arabinoxylan compared to F1. *In vitro* analysis of arabinose, xylose and wheat bran, and psyllium arabinoxylans have demonstrated that there is an increase in propionate production compared to butyrate, which may explain the lack of difference in propionate proportion in each of the fractions (Demuth, et al., 2021; Gietl, et al., 2012; Pollet, et al., 2012). This high relative propionate proportion could also be of benefit whilst preventing increases in gastric discomfort. When comparing the gas: propionate production ratio (supplementary file 2). F2 and F3 had a ratio of 1.04 (0.27) and 0.34 (0.16)

respectively indicating that for every mM of propionate produced ~ 1.04 or 0.34 ml of gas was produced. This may be of benefit when considering methods of increasing propionate production in the colon, whilst reducing gastric discomfort in conditions such as IBS.

Unlike for butyrate, which starch has been demonstrated to preferentially lead to the production of, substrates that preferentially produce propionate are less clear (Warren, et al., 2018). The psyllium fractions are likely of benefit as propionate production within the colon has been demonstrated to have many beneficial roles on maintaining gut health, in addition to other benefits within the body (reviewed elsewhere (Byrne, et al., 2015)).

3.3 Microscopy

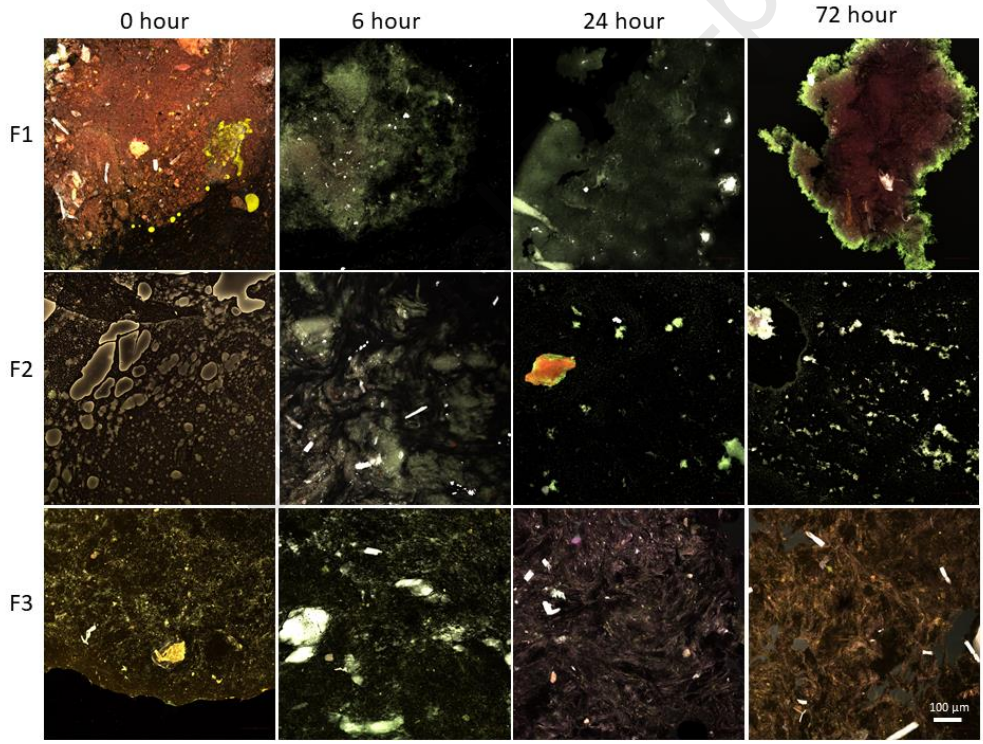


Figure 3 The substrate breakdown at time points 0, 6, 24 and 72 hours. Images are an overlay of all probes used. Images taken on a 10x objective.

Figure 3 shows the substrate breakdown and the bacterial interactions of the different psyllium fractions at 0, 6, 24 and 72 hours. In general, the colonic bacteria localised at the edge of the psyllium particles. Different bacteria did behave differently, Bacteroidetes (Bac303, Cyan) and Lactobacilli

(Lab158, orange) spread throughout the gel particles, in contrast Bifidobacteria (Bif164, yellow) did not as extensively co-localise to the psyllium and clustered at the edges of the gel particles.

The structure of the F1 fraction was disrupted by 6 hours of fermentation, additionally it can be seen that at 72 hours the bacteria distinctly localise to the edge of the psyllium gel particles. The structure of F2 was not disturbed to the same extent as F1, but by 24 hours the gel had dispersed as demonstrated by the lack of clear psyllium gel particulates. Notably, at 0 hours globules of psyllium stained with calcofluor white were observed. Dispersal of the gel was apparent for F3, globules similar to those found in F2 were also seen. By 72 hours the gel and bacteria were dispersed. The bacteria seem to be encapsulated in the husk sample. Despite yielding similar SCFA to F2 and F3, there is little apparent degradation in the structure of the husk. In contrast to (Yu, et al., 2017), distinct differences in the staining of the fractions were less clear. Within their study they observed that F2 was preferentially stained by calcofluor white (binds to β1,3 and β1,4 linked polysaccharides) and F3 preferentially by direct red (prefers to bind to β1,4 linked polysaccharides). In these study, we did not observe marked differences in the affinity of the two stains. This may be due to Yu et al. 2017 imaging the fractions under different conditions to those used in the present study. Yu et al. viewed the fractions dispersed in water without homogenisation, whereas in the present study the samples we dispersed in a complex media, homogenised and then cross-linked and embedded prior to imaging to fix the microbial communities.

4.0 Conclusion

Our results demonstrate that the viscosity of fibre ~~can~~ directly impacts gas production during fermentation, independently of sugar composition or linkage. In contrast, the viscosity plays a smaller role than the sugar structure and linkage when determining SCFA production. These data provide potential new avenues for the treatment of gastrointestinal disorders through a better control over gas and SCFA production in the colon by manipulating the hydrocolloid behaviour of fibre components.

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Highlights

- Psyllium fractions have distinct viscoelastic properties which affects how they are utilised in the gut
- Viscoelastic properties are important in determining colonic gas production
- Chemical properties are important in determining colonic short chain fatty acid production
- Psyllium fractions could be used to provide the health benefits of dietary fibre, whilst reducing gastric discomfort

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