Received: 9 November 2022 | Accepted: 9 March 2023

DOI: 10.1111/1462-2920.16368

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Beyond purified dietary fibre supplements: Compositional variation between cell wall fibre from different plants influences human faecal microbiota activity and growth in vitro

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Funding information
Scottish Government Rural and Environment Science & Analytical Services; University of Aberdeen.

Abstract

Dietary fibre is a major energy source for the human gut microbiota, but it is unclear to what extent the fibre source and complexity affect microbial growth and metabolite production. Cell wall material and pectin were extracted from five different dicotyledon plant sources, apples, beet leaves, beetroots, carrots and kale, and compositional analysis revealed differences in the monosaccharide composition. Human faecal batch incubations were conducted with 14 different substrates, including the plant extracts, wheat bran and commercially available carbohydrates. Microbial activity was determined for up to 72 h by measuring gas and fermentation acid production, total bacteria (by qPCR) and microbial community composition by 16S rRNA amplicon sequencing. The more complex substrates gave rise to more microbiota variation compared with the pectins. The comparison of different plant organs showed that the leaves (beet leaf and kale) and roots (carrot and beetroot) did not give rise to similar bacterial communities. Rather, the compositional features of the plants, such as high arabinan levels in beet and high galactan levels in carrot, appear to be major predictors of bacterial enrichment on the substrates. Thus, in-depth knowledge on dietary fibre composition should aid the design of diets focused on optimizing the microbiota.

INTRODUCTION

Improving health by modulation of the human gut microbiota via dietary manipulation has gained a lot of public interest over the last 10–15 years. Dietary fibre is a major energy source for the human gut microbiota with an estimated 14–25 g arriving in the large intestine daily in western societies (Stephen et al., 2017), whereas in rural African and hunter-gather societies, it ranges from 50 to 150 g/day (Hudson & Englyst, 1995; O'Keefe et al., 2015; Pontzer et al., 2018). Not a single country in Europe or North America meets their own recommended daily intake (Stephen et al., 2017) and the 2017 Global Burden of Disease study found that "a suboptimal diet is responsible for more deaths than any other risks globally, including tobacco smoking" (Afshin et al., 2019). The study found, perhaps surprisingly, that the lack of certain foods (whole grains, fruit, nuts & seeds and vegetables) rather than the overconsumption of unhealthy foods (processed meat and sugared sweetened beverages) together resulted in the highest number of deaths. Five out of the top seven contributors were either the lack of food groups rich in fibre or the lack of dietary fibre itself (Afshin et al., 2019). There

is therefore an urgent need to improve fibre intakes, however, dietary fibre is a group of molecules and molecular aggregates that differ greatly in their mono- and polysaccharide composition, hydration properties, solubility and viscosity, key factors affecting digestion and absorption in the upper gut and the consequent fermentation by the large intestinal microbiota (Flint et al., 2012). In the pursuit of closing the "fibre gap" the amount of fibre is obviously important, but a key question is: how important are composition and physicochemical properties of the fibre for gut health? In other words, can we close the "fibre-gap" by consuming manufactured food supplements (such as inulin or pectin), or should we eat a variety of whole plants foods?

To date, the majority of research investigating the effect of dietary fibre on the gut microbiota has focused on purified carbohydrates (Gibson et al., 2017) and less is known on how complex insoluble fibre affects bacterial growth and community composition (de Paepe et al., 2019; Puhlmann & de Vos, 2022). The cell wall composition varies between plants, in particular between grains (type II, rich in arabinoxylan and β-glucan) and non-grains (type I, rich in pectin and xyloglucan) (Fry, 2018). A recent in vitro study comparing 22 different fibre sources showed that grain fibre tended to produce more similar bacterial communities than fibre from pulses, seeds, and fruit (Calatayud et al., 2021). As the researchers only measured total dietary fibre and not the fibre composition, it is difficult to assess how compositional differences between the plants affected the bacterial communities.

Different microbes in the gut vary in their ability to degrade the different components of dietary fibre and extensive cross-feeding interactions take place, both of carbohydrate breakdown intermediates as well as fermentation products such as lactate (Louis et al., 2021; Louis et al., 2022). This ultimately leads to the production of mainly three organic fermentation products, the short-chain fatty acids acetate, propionate and butyrate, which exert health-promoting effects on the human host (Blaak et al., 2020). Several bacteria belonging to the phylum Firmicutes are nutritionally specialized, such as *Ruminococcus bromii* for resistant starch (Ze et al., 2012), *Ruminococcus champanellensis* for cellulose (Chassard et al., 2012) and *Lachnospira eligens* (recently reclassified from *Eubacterium eligens* (Oren & Garrity, 2020)) for pectin (Chung et al., 2017). Many Bacteroidetes utilize a wider variety of fibre sources, which is reflected in their large number and varied repertoire of carbohydrates active enzymes (Ndeh & Gilbert, 2018). Cell wall polysaccharides in whole plants are covalently linked (Fry, 2018; Zykwinska et al., 2005). Substantial amounts of the soluble pectin and hemicellulose constituents might therefore be bound up in the cell wall matrix upon arrival in the large intestine, and consequently inaccessible to many gut microbes, as the liberation of cell wall polysaccharides likely depends on primary degraders such as *R. champanellensis*, which has both xylanolytic and cellulolytic activity (Chassard et al., 2012). A dietary fibre supplement containing purified cell wall constituents might therefore have a different effect on the microbiota than if eaten as a part of a whole plant-based diet and there is a need to better understand how the physicochemical characteristics and compositional differences of dietary fibre from different plants affect microbial growth, community interactions and metabolite formation.

When preparing fibre material for in vitro experimentation, it should be as close as possible to its natural state after passing through the upper gut in vivo. It is therefore important to use mild methods which lead to little material degradation. The preparation of alcohol-insoluble residue (AIR), where the plant material is suspended in ethanol to remove simple sugars and certain proteins, is a mild and easy way to produce a cell wall-rich pellet (Fry, 2018). AIR may also contain some protein and starch, however, their amounts can be quantified, and if low, the material can be used directly as a growth substrate without the need for in vitro upper gut digestion, as it mainly consists of cell wall polysaccharides. Plant polysaccharides that are commercially available are often extracted using harsh methods, which can result in alteration and breakdown of the material and consequently affect bacterial fermentation and growth (Fry, 2018; Koubala et al., 2008; Larsen et al., 2019). The aim of this study was to investigate how dietary fibre composition and complexity affects human gut microbial communities, by preparing AIR and pectins from different plants and plant organs and assessing their effects on growth and activity of human faecal microbiota during in vitro batch incubations.

EXPERIMENTAL PROCEDURES

Preparation of plant material

Organically farmed apples (*Malus Domestica* variety Fiesta), beet leaves, beetroots (both *Beta vulgaris* variety Robuschka), carrots (*Daucus carota* variety Rodelika) and kale (*Brassica oleracea* variety Sabellica) were bought from Vital Veg (www.vitalveg.co.uk, Aberdeenshire) in early December 2019 and processed within 3 days of purchasing. The wheat bran was a gift from Dr. Sylvia Duncan and was already prepared as AIR as previously described (Duncan et al., 2016, designated as coarse material below). The fresh produce was washed, weighed and freeze-dried (Multidry, Frozen in Time Ltd, York, UK). The dry material was weighed and freezer-milled (6870 SPEX sample prep, Middlesex, UK) in liquid nitrogen (cool-down period of 15 min; milling 2 min; cool down 1 min; milling 2 min). The samples were vacuum-packed and stored as dry

powder at −70°C. Dry matter content was calculated by the ratio of dry to fresh weight using the following equation: Percentage dry matter = (dry weight [g] × 100)/fresh weight [g].

For preparation of alcohol-insoluble residue (AIR), the alcohol-soluble portion of the plant was removed by stirring the powdered plants (33.3% [w/v]) in 30 ml 70% ethanol at room temperature for 3 days (until the supernatant was colourless). The 70% ethanol was replaced in the morning, noon, and late afternoon, by centrifugation (3000 *g*, RT, 5 min; Eppendorf 5810R, Stevenage, U.K) and removal of the supernatant. The AIR was freeze-dried (Labconco, Kansas City, USA), weighed and stored at room temperature. The ratio of AIR to dry plant powder was calculated as follows: % AIR = (AIR [g] × 100)/dry plant powder [g]. Enzymatic removal of starch and protein from AIR were not carried out to limit heating and drying and produce a substrate with as intact a cell wall matrix as possible for the downstream bacterial fermentation experiments.

Quantification of protein, starch and ash in AIR

Routine proximate analysis was carried out to determine the content of starch, protein and ash in the AIR. Starch was quantified as described by Aaman and Hesselman (1984). Briefly, starch was digested using a combination of heat-stable α-amylase and amyloglucosidase. The released glucose was measured spectrophotometrically (560 nm; μQuant Plate Reader, Biotek, Vermont). Protein was measured as nitrogen by the Dumas combustion method (Etheridge et al., 1998) using a Vario Max CN analyser. The nitrogen was converted to protein by the conversion factor of 5.6 (Mariotti et al., 2008). For ash quantification, 2.5–3 g AIR was placed in a muffle furnace (LT 24/11, Nabertherm, Lilienthal, Germany) for a minimum of 3 h at 550°C. The cooled sample was weighed and the percentage ash calculated: %ash = (weight ash × 100)/(weight AIR aliquot). As the quantification of ash required large amounts of sample and the amount of AIR was limited, only a single replicate was analysed, whereas starch and protein were analysed in triplicate.

Quantification of cell wall polysaccharides in AIR

AIR was hydrolyzed for 1 h at 121°C on a heat block with 2 M trifluoroacetic acid (TFA) as described by Saez-Aguayo et al. (2017). The TFA was evaporated at 45°C under a flow of nitrogen and samples were rinsed twice with isopropanol. The samples (monosaccharides from hemicellulose and pectin) were resuspended in 600 μl MilliQ water, cleaned by passing through a syringe filter (0.22 μm), transferred to an HPLC glass vial and analysed according to Saez-Aguayo et al. (2017) using a Dionex ICS3000 ion chromatography system (Sunnyvale, USA), equipped with a pulsed amperometric detector. Myo-inositol and allose were used as internal standards (250 μM each). The weight of the remaining pellet (cellulose) was determined gravimetrically. Uronic acids were quantified colourimetrically according to the Englyst NSP method (1988).

Pectin extraction and monosaccharide composition analysis

Pectin was prepared from AIR of apple, beet leaf, beetroot, carrot and kale by incubation with 0.2 M ammonium oxalate (pH 4.3) as previously described (O'Rourke et al., 2015). Two additional extractions were added to the protocol to achieve complete extraction, giving four sequential extractions: (1) 60°C for 16 h (to avoid breakdown via β-elimination), (2) 100°C for 2 h, (3) 100°C for 16 h, and (4) 100°C for 16 h. Pectin was prepared on two separate occasions (pectin I and pectin II). For pectin I, the composition of the neutral monosaccharides was determined by HPLC after hydrolysing the samples with TFA as described above. Uronic acids were determined with the m-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) modified to 96-well plate format. For pectin II, the monosaccharide composition for each sequential extraction was determined. The pectins were hydrolysed with TFA as described above and the neutral monosaccharides were determined by GC according to Englyst and Cummings (1988). The protocol was followed from the reduction step onwards, as the starting material was pure pectin and not AIR (i.e. steps for removal of protein and starch were unnecessary). The acidic monosaccharides (uronic acid) were determined according to Englyst and Cummings (1988) as above, however, the starting aliquot was a solution of pectin (1 mg/ml) instead of an aliquot from the AIR hydrolysate. Commercially available apple and citrus pectin were obtained from Sigma-Aldrich, Poole, UK for comparison to in-house extracted pectins.

Analysis of the methylation degree of pectin

The methylation degree was calculated from the amount of methanol released from the pectin (50 μl; 1 mg/ml) by alkali de-esterification with 0.2 M NaOH (1 h, 50 μl; 4°C on ice). The solution was neutralized with 0.2 M HCl (50 μl) and the methanol quantified as previously described by Ralet et al. (2012).

In vitro fermentations

Anaerobic incubations were carried out in Hungate tubes as described previously (Reichardt et al., 2018). Ethical approval for the study was granted by the internal Rowett Institute Ethical review panel. Fresh faecal samples were obtained from three different donors (two female and one male, age range 33–59, D1–D3) with no history of gastrointestinal disorders or antibiotic treatment for at least 3 months prior to the study and processed within 2–3 h after defecation. Two grams of each individual faecal sample were diluted 5-fold with 8 ml of anaerobic phosphate buffered saline (Sigma-Aldrich, Poole, UK) and homogenized in a gentleMACS C tube (Miltenyi Biotech, Surrey) on a Dispomix Drive (Medic Tools, Lussiwag, Switzerland). The homogenate was further diluted 5-fold in anaerobic phosphate buffered saline and 0.5 ml was used as an inoculum for 9.5 ml of fermentation medium (Reichardt et al., 2018), set to pH 6, to achieve a final faecal concentration of 0.2%. Substrates containing a mixture of dietary fibre carbohydrates, including poorly fermentable cellulose, were incubated at 0.6% (w/v). They included in-house AIR preparations and a positive growth control consisting of a mix of different carbohydrates to simulate the major components of plant cell walls (recombined cell wall, 'RCW'), consisting of 33.33% cellulose (11365, Sigma Aldrich), 33.33% apple pectin (76282, Sigma Aldrich), 22.11% xyloglucan (P-XYGLN, Megazyme), 9.11% xylan (P-XYLNBE, Megazyme) and 2.11% mannan (P-MANCB, Megazyme). In-house pectin extractions were incubated at 0.2%, as pectin approximately makes up one third of the cell wall (Fry, 2018), and inulin (Sigma Aldrich I2255; 0.2%) was included to represent a purified soluble fibre and as it is one of the most studied prebiotics (Gibson et al., 2017). Control incubations with no added substrate ('NO CHO') were run in parallel to establish microbial activities on the basal medium ingredients. All substrates were sterilized in absolute ethanol and dried under a flow of nitrogen.

Incubations were carried out in triplicate at ~90° angle on a rotator (Stuart SB3, Bibby Scientific, Stone, UK) at 25 rpm, 37°C. Gas production was measured after 24, 48 and 72 h with a glass syringe by penetrating the rubber seal of the Hungate tube with a sterile needle to enable pressure equilibration to atmospheric pressure and recording the displayed volume. Then, culture aliquots (1.5 ml) were removed from the incubation tubes under CO_2 at 24, 48 and 72 h. The aliquots were centrifuged (10,000×*g*; 10 min; 4°C; miniSpin, Eppendorf, Stevenage, UK) and the supernatant collected for short-chain fatty acid (SCFA) analysis. The pellet was resuspended in sodium phosphate buffer (978 μl) and MT buffer (122 μl), both part of the FastDNA spin kit for soil (MP Biomedicals, Illkirch, France), and stored at −70°C until DNA extraction. On the day of inoculation ($T = 0$ h), 500 μl aliquots of the faecal inoculum were added to lysis matrix tubes together with sodium phosphate buffer (478 μl) and MT buffer (122 μl) and stored at −70°C until DNA extraction.

SCFA analysis

SCFA concentrations were measured in 0.5 ml culture supernatants using gas chromatography as described previously (Richardson et al., 1989). After derivatisation, 1 μl of sample was analysed using a Hewlett-Packard gas chromatograph fitted with a silica capillary column with helium as a carrier gas. The SCFA concentrations were calculated from the relative response factor with respect to the internal standard 2-ethylbutyrate.

DNA extractions and qPCR

DNA extractions were performed with a FastDNA spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA concentrations were measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Paisley, UK). The total number of 16S rRNA gene copies per ml of faecal incubation was determined by quantitative PCR (qPCR) as described previously (Reichardt et al., 2018) with universal primers (UniF, GTGSTGCAYGGYYGTCGTCA; UniR, ACGTCRTCCMCNCCTTCCTC; 500 nM each) and 2 ng DNA in a total volume of 10 μl and expressed as 16S rRNA gene copies per ml of culture.

16S rRNA amplicon Illumina sequencing

Sequencing was performed at the Centre for Genome Enabled Biology and Medicine (CGEBM, University of Aberdeen). PCR amplification (25 cycles) of extracted DNA from the anaerobic faecal incubation experiments as template was carried out using universal primers targeting the V1/V2 variable regions of the 16S rRNA gene (OH27F: 5′ TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGM GTT YGA TYM TGG CTC AG 3′; OH338: 5′ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC CTC CCG TAG GAG T 3′). All amplicons were pooled alongside positive (PhiX control) and negative (molecular biology water) control and sequenced on a single Illumina MiSeq v3 flowcell, producing 2 × 300 bp paired-end reads.

The raw Illumina data were inspected with FastQC (Andrews, 2010; Galaxy Version 0.72) and Trim Galore (Krueger, 2012; Galaxy Version 0.6.3; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; Accessed October 16, 2021) was used to remove primers and reads with a Q-score (Phred quality score for base calling accuracy) below 30. Further quality

trimming, denoising of sequence errors, assembly to full-length amplicon sequence variants (ASVs) and chimera removal was carried out using the DADA2 pipeline (Callahan et al., 2016) (version 1.14.1) in R (R core Team, 2021; version 3.6.2). Based on quality plots, forward and reverse reads were trimmed to 190 bp. Denoising of sequence errors and assembly to full-length amplicon sequencing variants (ASVs) resulted in 3397 ASVs across a total of 219 samples (all 48 h samples, a selection of 24 h samples and nine samples belonging to another study). Assembled ASVs shorter than 300 bp and with two or more expected errors or unresolved bases were removed (Edgar & Flyvbjerg, 2015). Chimeras were removed, leaving behind 2093 ASVs accounting for 98.4% of the paired reads. Taxonomy was assigned to the ASVs using SILVA database nr 99 version 138 (Yilmaz et al., 2014). ASVs that were annotated as chloroplast, mitochondria, archaea or eukaryota were removed, resulting in 1794 ASVs across the 210 samples belonging to this study and library sizes of 1165–46,573 (average 18,660) reads (Table S1). The libraries were rarefied to even depth to ensure an equal comparison between samples. The individual ASVs were phylotyped at the genus level, as ASV level data are difficult to interpret with a high level of unclassified ASVs at species level (78% in this dataset).

Alpha and beta diversity at the genus level were calculated using the R packages Vegan (Oksanen et al., 2020) and Phyloseq (McMurdie & Holmes, 2013). Shannon index (Shannon, 1948) was used to assess alpha diversity and Bray Curtis dissimilarity (Bray & Curtis, 1957) to assess beta diversity. Principal coordination analysis (PCoA) plots were used to visualize the Bray Curtis dissimilarity and statistical differences were assessed by permutational multivariate analyses of variance (PERMANOVA) using the Adonis function in the Vegan package in R. The linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) was used to evaluate differentially abundant genera, using the default settings (α parameter ≤0.05 for both class normality and subclass tests). The p-values were adjusted for false discovery rates with the Benjamini–Hochberg method (Benjamini & Hochberg, 1995), q value = 0.05. The sequencing data generated in this study have been deposited in the National Centre for Biotechnology Information sequence read archive (NCBI SRA) under accession numbers PRJEB48569.

Statistical analysis

Independent samples t tests and one-way ANOVA (analysis of variance) were conducted in SPSS 25.0 (IBM, Chicago, USA) and a p value ≤0.05 was considered statistically significant for all analyses. In the few cases where the normality of the data could not be assumed, they were log-transformed (base 10) to obtain normality. For ANOVA, comparison between treatment groups and adjustment for multiple testing was done with Tukey's post hoc tests, where the assumption of homogeneity of variances was met and Games-Howell post hoc tests were done where homogeneity of variance was not met. The following assumptions were tested and met to justify the use of ANOVA to test for the significance between the means of two or more independent groups: (1) Normality of residuals: This was assessed by histograms and Quantile-Quantile plots (Q-Q plots) of the standardized residuals compared with the normal distribution of the standardized residuals and (2) Homogeneity of variance: Levene's test was used to assess that the variance of all data points of the dependent variable was constant for each group.

Principal Components Analysis was conducted in R using the 'prcomp' function. Data analysis and graphical representations, which were not done in R or SPSS, were conducted in Microsoft Excel.

RESULTS

Composition of plant-derived substrates

Composition of alcohol-insoluble residue (AIR) preparations

AIR was prepared from five different dicotyledon plant organs, apple fruit ('apple'), carrot root ('carrot'), beetroot, beet leaf and kale leaf ('kale'), allowing for comparisons between plants and plant organs. Wheat bran AIR was included as a representative monocotyledon plant. The amount of dry matter in the fresh plants ranged from 12.6% (beet leaf) to 18.0% (beetroot) (Figure S1A). AIR ranged from 15% to 57% of the dry plant material, with leaves containing the highest amount (Figure S1B). The total relative amount of non-fibre components (sum of protein, starch, ash) in AIR was lowest in apple (9.6%) and highest in beet leaf (33.9%) (Figure 1A). Carrot and wheat bran were the only AIR samples with detectable, but low, amounts of starch (1.6 ± 0.1% and 0.9 ± 0.1%, respectively). Protein content was highest in beet leaf AIR (27.9 ± 0.1%) and lowest in apple AIR (4.6 ± 0.1%), whereas the amount of ash ranged from 4.5% (carrots) to 9.5% (kale) (Figure 1A). Overall non-fibre components constituted a relatively small fraction of the AIR preparations, but co-precipitated protein may contribute to microbial fermentation for some substrates (in particular beet leaf and kale).

The AIR carbohydrate composition was determined by hydrolysis of non-cellulosic polysaccharides with TFA and gravimetric quantification of cellulose. The

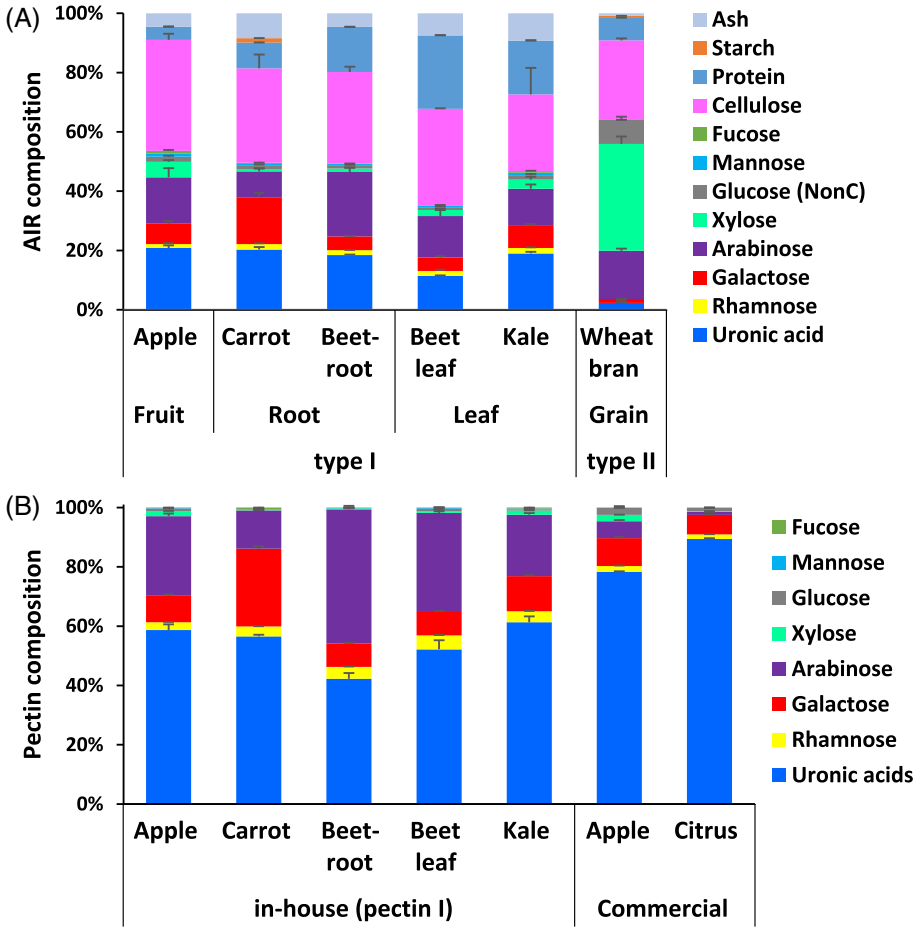

FIGURE 1 Relative monosaccharide composition of substrates prepared from fresh plants for microbial incubations (for absolute data see Figure S1). (A) Composition of AIR preparations. Plant organ and cell wall type are indicated. Glucose originates from non-cellulosic origin (NonC). Mean and standard deviation of triplicate analysis, apart from carrot cellulose ($n = 2$) and ash across samples ($n = 1$). (B) Composition of pectin preparations from type I plants and pectins from two commercial sources. Mean and standard deviation of triplicate analysis.

total recovery based on the sum of the individual components ranged from 784.7 to 1107.5 mg/g of dry AIR (Figure S1C). The relative amount of the more readily fermentable fibres (i.e. hemicellulose and pectin monosaccharides) ranged from 35.2% in beet leaf to 65.7% in wheat bran (Figure 1A). As expected, the most distinct compositional difference was observed between wheat bran (type-II cell wall, xylose and glucose significantly ($p < 0.001$) more abundant) and the non-grain samples (type-I cell walls, significantly higher ($p < 0.01$) in pectin constituents uronic acids and rhamnose) (Figures 1A and S1C). Colourimetric determination of uronic acids measures both glucuronic and galacturonic acids, however glucuronic acid was present in only minor amounts as determined by HPLC (average across samples: 2.5 ± 1.5 mg/g dry AIR, data not shown). Within type-I cell wall substrates, beet leaf had significantly ($p < 0.01$) lower relative amounts of uronic acids (11.4 ± 0.2%) compared with the other samples (average across samples 20.0 ± 1.1%). Carrot AIR had significantly higher ($p < 0.001$) relative amounts of galactose compared with the other samples and was the only sample with higher amounts of galactose than arabinose. Beetroot displayed a significantly ($p < 0.001$) higher amount of arabinose compared with the other type-I cell wall samples.

Composition of pectin preparations

Pectin was extracted from the five dicotyledon plants on two separate occasions from the same starting material (pectin I and II). The total yield matched well between pectin I and II (Figure S2A), with the highest amounts being found in kale (189.2 ± 1.9 mg/g dry plant) and the lowest in apple (41.8 ± 4.4 mg/g dry plant). Four extractions appeared to be sufficient to extract the majority of pectin from the dry plant, as the first three extractions for pectin I accounted for 88.8 ± 6.3% of the total pectin across all plants. However, some variation was seen between the plants in terms of yield from each extraction, with both beet leaf and beetroot displaying higher relative yields in the two latter extractions for both pectin I & II (45.9 ± 12.3%)

compared with apple, carrot, and kale (24.0 ± 8.2%). This suggests more cross-linking between the pectins and the other cell wall constituents in beetroot and beet leaf, making the material harder to extract.

The monosaccharide composition was determined for the sequential pectin II extractions, which showed a significant ($p < 0.001$) stepwise decrease in the relative amount of uronic acids together with an increase in other monosaccharides (Figure S2B). Assuming uronic acids as a proxy for homogalacturonan, and arabinose, galactose and rhamnose as proxies for rhamnogalacturonan I (RG-I), this indicates that pectin molecules that are richer in homogalacturonan are more easily extractable than pectins rich in RG-I, likely due to more cross-linking between RG-I side chains and the other cell wall polysaccharides (Sibakov et al., 2013; Wieser et al., 2020). The sequential pectin extractions were pooled and the monosaccharide composition analysed as complete pectin. Pectin I and II showed similar compositions and broadly mirrored the results from the total AIR preparations, in particular the high galactose content in carrot pectin (Figure S2A). Pectin I preparations were used for microbial incubations and their composition was therefore compared with commercially available apple and citrus pectin. The in-house pectins had a significantly ($p < 0.001$) lower relative amount of uronic acids (55.0 ± 5% vs. 84.3 ± 5.1%, respectively) and higher relative amount of galactose plus arabinose compared with the commercial pectins (40.3 ± 7.9% vs. 11.1 ± 5.9%, respectively) (Figure 1B). Between the in-house pectins, beetroot and beet leaf displayed significantly ($p < 0.05$) lower relative amounts of uronic acids and higher ($p < 0.001$) amount of galactose plus arabinose compared with pectin from apple, carrot, and kale, which was further reflected in the ratio of arabinose plus galactose to uronic acids (Table 1). Carrot pectin had a significantly ($p < 0.001$) higher relative amount of galactose compared with the other in-house pectins and was the only in-house pectin with higher levels of galactose than arabinose (Ara:Gal ratio 0.5). At the other extreme were the beet samples, both of which had significantly ($p < 0.001$) higher relative amounts of arabinose than the other pectins (Figure 1B) and an Ara:Gal ratio of around 5 (Table 1). Rhamnose, which is part of the RG-I backbone, was present at a significantly ($p < 0.001$) higher relative amount in the in-house pectins (3.7 ± 0.8%) compared with the commercial pectins (1.9 ± 0.3%). The non-pectin monosaccharides mannose and glucose together accounted for <1.5% across the in-house pectins, indicating that the pectins were pure. The degree of methylation ranged from 21.5 ± 0.3% in carrot to 46.5 ± 1.5% in commercial apple pectin. Although there were significant ($p < 0.001$) differences in the degree of methylation between the samples (Table 1), it was below 50% in all samples and they were consequently considered to have a low degree of methylation (cutoff 50%; Mudgil, 2017).

Microbial activity and growth during faecal batch fermentations on different substrates

Bacterial activity as determined by gas production over 72 h of incubation

Microbiota batch fermentations with faeces from three individual healthy human donors (D1-D3) were carried out over 72 h with the different plant preparations. Wheat bran was prepared both as a freeze-milled powder and as coarse material to investigate the role of particle size. A "recombined" type-I cell wall substrate (RCW, for details see experimental procedures) and inulin were included as positive growth controls. A no carbohydrate ('NO CHO') control was included to establish microbial activities on the basal medium ingredients. Gas production was used to measure bacterial activity during incubation after 24, 48 and 72 h. For most incubations the highest gas production

TABLE 1 Structural characteristics of in-house pectin I preparations compared with commercially sourced pectins from different plants.

Source	Plant	Monosaccharide ratio of		Degree of methylation (%)[b]
		Ara:Gal	Ara + Gal:Uronic acid[a]	
In house	Apple	2.9	0.6	37.5 ± 0.9[a]
	Carrot	0.5	0.7	21.5 ± 0.3[b]
	Beetroot	5.8	1.4	28.8 ± 0.6[c]
	Beet leaf	4.9	0.9	33.0 ± 0.3[d]
	Kale	1.7	0.6	26.9 ± 0.7[e]
Commercial	Apple	0.6	0.2	46.5 ± 1.5[f]
	Citrus	0.2	0.1	36.8 ± 0.8[a]

Abbreviations: Ara, arabinose; Gal, galactose.
[a]Significance ($p < 0.05$) for degree of methylation indicated by superscript letter, letters in common are not significantly different.
[b]Uronic acid originating from homogalacturonan was estimated by subtracting rhamnose from total uronic acid, as the RG-I backbone has 1:1 ratio of galacturonic acid to rhamnose.

occurred within the first 24 h, whereas very little or no gas was detected after 48 h, indicating that fermentation was largely complete at this stage (Figure 2). All donors produced a significantly ($p < 0.001$) higher amount of total gas from the growth substrates compared with the no-carbohydrate control and the three donors had a relatively similar response to the individual substrates, with a significantly ($p < 0.05$) higher amount of total gas being produced on AIR from apple, carrot, beetroot, kale and the RCW compared with beet leaf and the wheat brans. Beet leaf had the lowest relative amount of hemicellulose and pectin (Figure 1A) and fermentable fibre may also be less accessible in these substrates. All donors produced more gas on powdered compared with coarse wheat bran (only significant [$p < 0.001$] for D1 and D2) (Figure 2A). Furthermore, all donors produced approximately equal amounts of gas at both 24 and 48 h on beet leaf and wheat bran powder, suggesting a less extensive, more prolonged fermentation. The purified pectins and inulin displayed little variation in the total amount of gas produced across substrates and donors (Figure 2B), however, D3 produced significantly ($p < 0.05$) lower amounts of gas within the first 24 h than D1 and D2.

Bacterial growth based on the total number of bacterial 16S rRNA gene copies

As there was very little gas production detected after 48 h of incubation, bacterial growth and fermentation acid production were determined after 24 and 48 h. As several of the substrates used are insoluble, interfering with optical density measurements, the total number of 16S rRNA gene copies per ml (designated 'total bacteria' hereafter) was determined as a measure for overall community growth. Across the AIRs and the RCW, D2 and D3 did not display a significant change in total bacteria between the time points, indicating that the communities had largely finished growing by 24 h

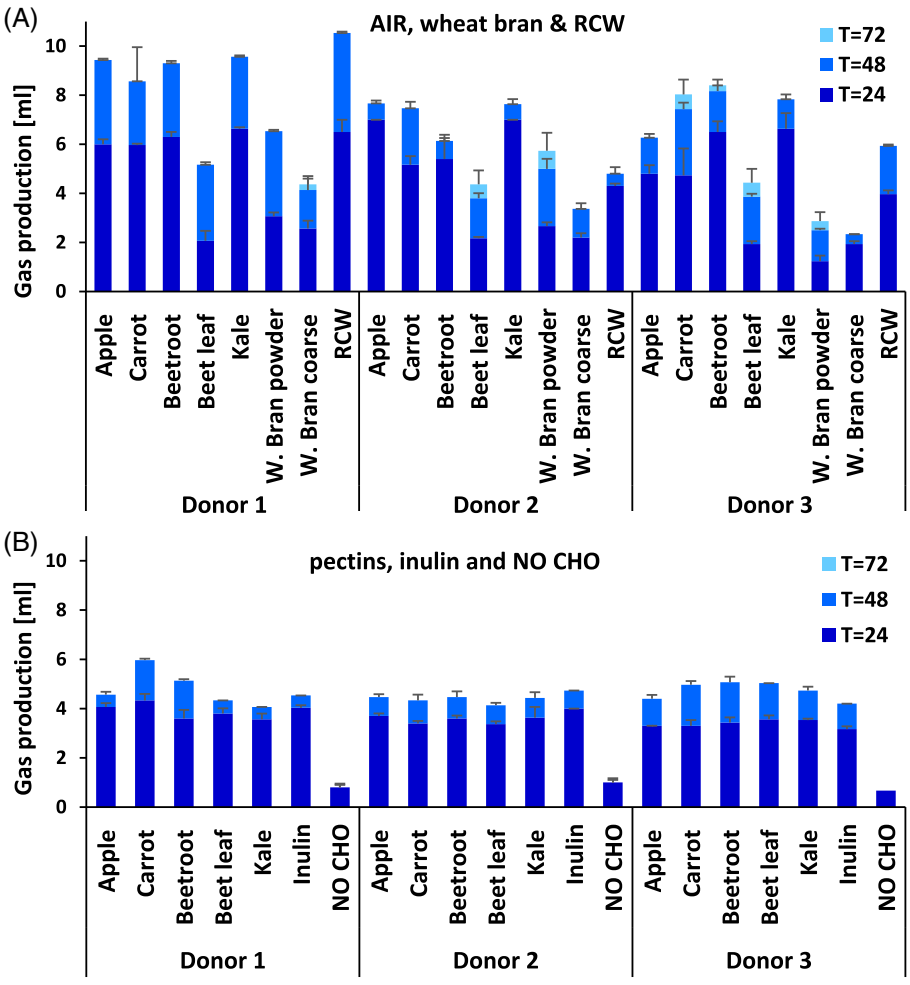

FIGURE 2 Gas production after 24, 48 and 72 h of incubation on 14 substrates and with faecal microbiota from three healthy donors (mean and standard deviation, $n = 3$; ANOVA and Tukey post hoc test results presented in the text). (A) Gas production on different plant cell wall preparations and a mix to different plant cell wall carbohydrates (RCW). W. Bran, wheat bran. (B) Gas production on pectin preparations from different plants, commercially sourced inulin and no carbohydrate control (NO CHO).

(Figure S3A). In contrast, D1 showed a significant ($p < 0.001$) decrease in total bacteria, suggesting bacterial lysis. Paradoxically, D1 produced significantly ($p < 0.001$) more gas from 24 to 48 h compared with D2 and D3, indicating higher bacterial activity. However, with the measurements being 24 h apart, peak gas production and peak total bacteria may not have coincided. On pectins and inulin, D1 and D3 also showed a significant ($p < 0.001$) decrease in total bacteria from 24 to 48 h (Figure S3B).

Fermentation acid production after 24 and 48 h of incubation

The three main fermentation products across all incubations at both time points were acetate, propionate, and butyrate (95.2 ± 5.0% of total fermentation acids), whereas formate was mainly present in incubations from D1 and D2 (Figure S4). Valerate, caproate, isobutyrate and iso-valerate were only detected in minor amounts, indicating little protein fermentation. Lactate and succinate were not detected in any of the donors. All incubations apart from D1 on beet leaf and D3 on the wheat brans produced significantly ($p < 0.05$) more total fermentation acids from the growth substrates compared with the no carbohydrate control at both timepoints. Differences between 24 and 48 h were minor, with D1 showing a tendency for a slight decrease and D2 and D3 for an increase across the substrates (Figure S4).

To compare substrates at different concentration levels (0.6% w/v AIR & RCW and 0.2% w/v pectins and inulin), a relative comparison between the fermentation acids at 48 h was conducted (Figure 3). D1 incubations displayed significantly ($p < 0.001$) higher relative butyrate amounts across substrates (26.1 ± 9.4%) than D2 (20.6 ± 5.9%) and D3 (18.7 ± 2.9%), which was accompanied by significantly ($p < 0.001$) lower relative amounts of acetate (47.6 ± 9.9%; D2 55.8 ± 6.8%; D3 57.4 ± 6.2%). D3 incubations showed higher relative amounts of propionate than the other two donors (only significant ($p < 0.001$) compared with D2). Formate also differed significantly ($p < 0.001$) between donors (D1 8.6 ± 4.6%; D2 5.0 ± 4.15%; D3 0.7 ± 1.1%). Significant differences between donors were also observed for different growth substrates (Figure 3 and Table S2). Wheat bran and inulin incubations resulted in high relative amounts of butyrate in D1 and D2 and course wheat bran material was higher than the powder. On the RCW, D1 and D3, but not D2, showed high relative amounts of butyrate. Between the type-I cell wall AIRs, all donors had the highest relative butyrate amounts for apple and carrot, whereas the beet leaf AIR resulted in significantly ($p < 0.05$) higher propionate amounts compared with the other AIRs for most incubations, with similar trends being observed for beetroot (Figure 3 and Table S2), possibly due to the high arabinan amounts in beet-derived AIR (Figure 1A). Compared with type-I AIRs, D1 and D2, but not D3, showed significantly ($p < 0.001$) lower relative butyrate amounts across the pectins. However, faecal incubations in the presence of different pectin preparations resulted in less variation of individual fermentation acids between the substrates (Figure 3).

Bacterial activity based on final medium pH after 72 h of incubation and comparison of all measures of community activity and growth

Measures allowing monitoring of microbial growth or activity during fermentation experiments that do not require sample processing and downstream analyses are desirable, in particular when optical density cannot be utilized. Medium pH can be used as a proxy for bacterial activity, and it was therefore measured at the end of the incubations (after 72 h) to assess how well it agrees with the other growth measures carried out here. The net pH change relative to the no carbohydrate control (NO CHO pH change from starting pH 6 across donors <0.2) was significantly ($p < 0.001$) larger in D2 faecal incubations compared with D1 and D3 on the pectins and inulin, and compared with D1 on the AIRs and the RCW (Figure S5), indicating that pH lowering did not become inhibitory to growth of D1 and D3 communities on substrates with low gas production such as wheat bran.

The four methods utilized here to assess microbial activity and growth overall showed strong correlations, with a correlation coefficient above 0.7 for 24-hour measures and generally above 0.6 for the 48-hour measures (72 h for pH) (Table S3 and Figure S6). The exception was D1 at 48 h for the correlation between total bacteria and the other measures, due to the drop in total bacteria between 24 and 48 h on some substrates. It is unlikely that this is due to technical issues, as all qPCR samples were processed together and also repeated for D1, which confirmed the initial results (data not shown). All other 48-hour correlations were strong for D1 (>0.8). Together, this shows that all different measures employed here are good indicators for bacterial growth and activity, but that total bacteria measures appear to be more sensitive, possibly due to changes of bacterial cells and their DNA once growth has ceased.

Microbiota community composition as determined by 16S rRNA gene sequencing

Overall microbial community characteristics after 48 h of incubation

The bacterial community composition was analysed by Illumina sequencing of the V1/V2 region of the 16S

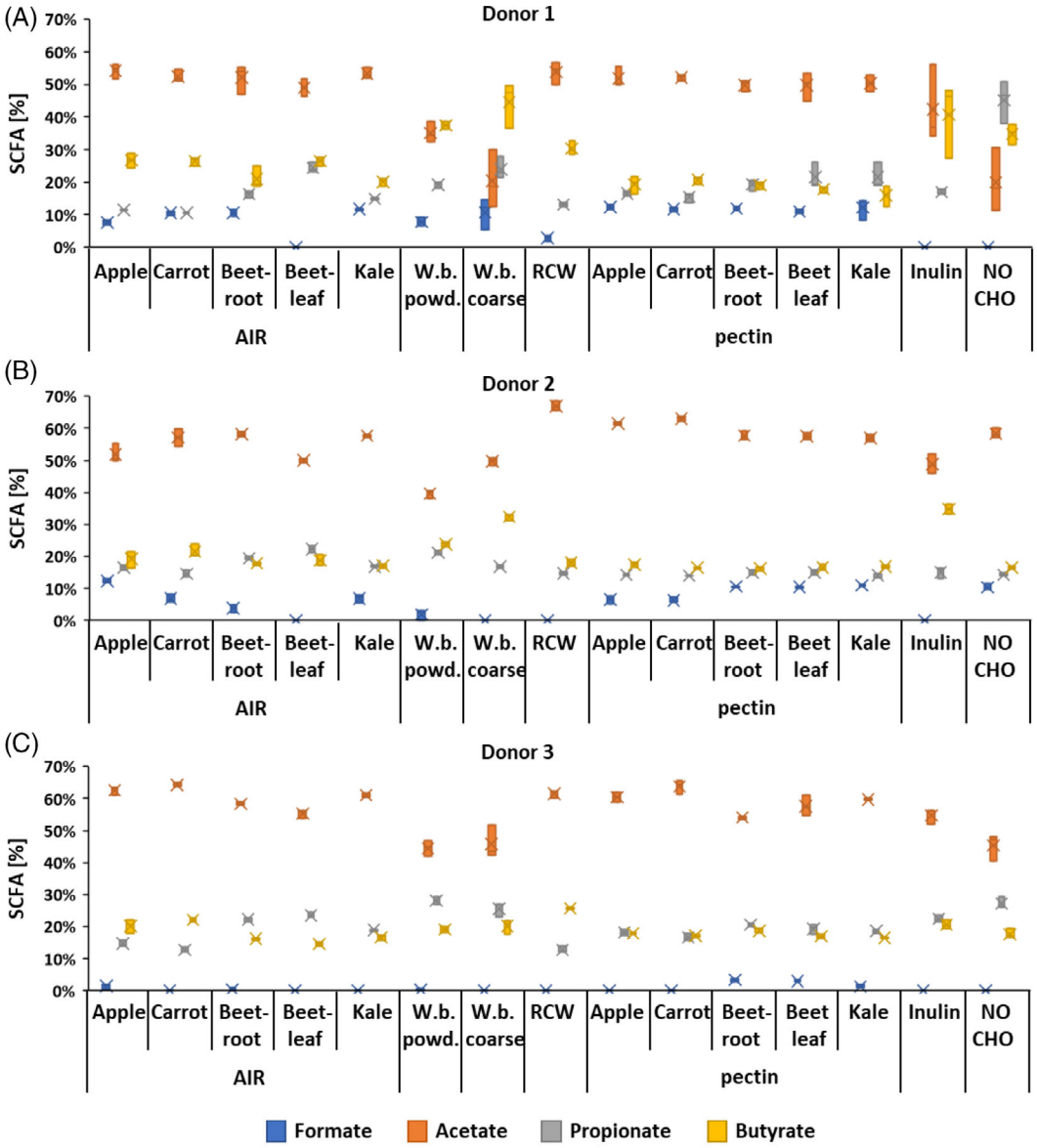

FIGURE 3 Box plot of net amount of formate, acetate, propionate, and butyrate relative to total fermentation acids after 48 h of incubation on 14 substrates and no carbohydrate control (NO CHO) with faecal microbiota from three healthy donors. Boxes ($n = 3$) extend to maximum and minimum and crosses indicate mean values. Post hoc significance tables are provided in Table S2. W.b., wheat bran; powd., powder.
(A) Relative SCFA production for D1. (B) Relative SCFA production for D2. (C) Relative SCFA production for D3.

rRNA gene. The most dominant genera in the faecal inocula were *Bacteroides*, *Faecalibacterium* and *Blautia* in all three donors, but differences were apparent between donors, in particular a high relative abundance of *Blautia* in D3 (Figure 4B, 'Inoculum'). As a substantial amount of bacterial activity was observed based on gas production between 24 and 48 h for some substrates, microbial community composition was assessed for all incubations after 48 h of incubation. A selection of incubations were also sequenced after 24 h, which mostly revealed little variation between the time points (Figure S7). The largest differences were seen in D1 on AIR substrates, in line with the drop in total bacteria based on qPCR.

The two main phyla detected across all substrates and donors after 48 h were Bacteroidetes and Firmicutes, with Proteobacteria and Actinobacteria also being detected (Figure S8). ASVs were assigned to 147 genera, with the top 20 accounting for an average of 82.6 ± 7.0% of the bacterial abundance across donors and substrates (Figure 4). The four most dominant genera across substrates and donors were *Bacteroides*, *Lachnospira eligens* group, *Faecalibacterium* and *Blautia*. There were relatively large compositional differences between the AIRs, both within and between donors, indicating that both substrate and faecal donor had a relatively large impact on the community composition, whereas the pectins gave rise to more similar

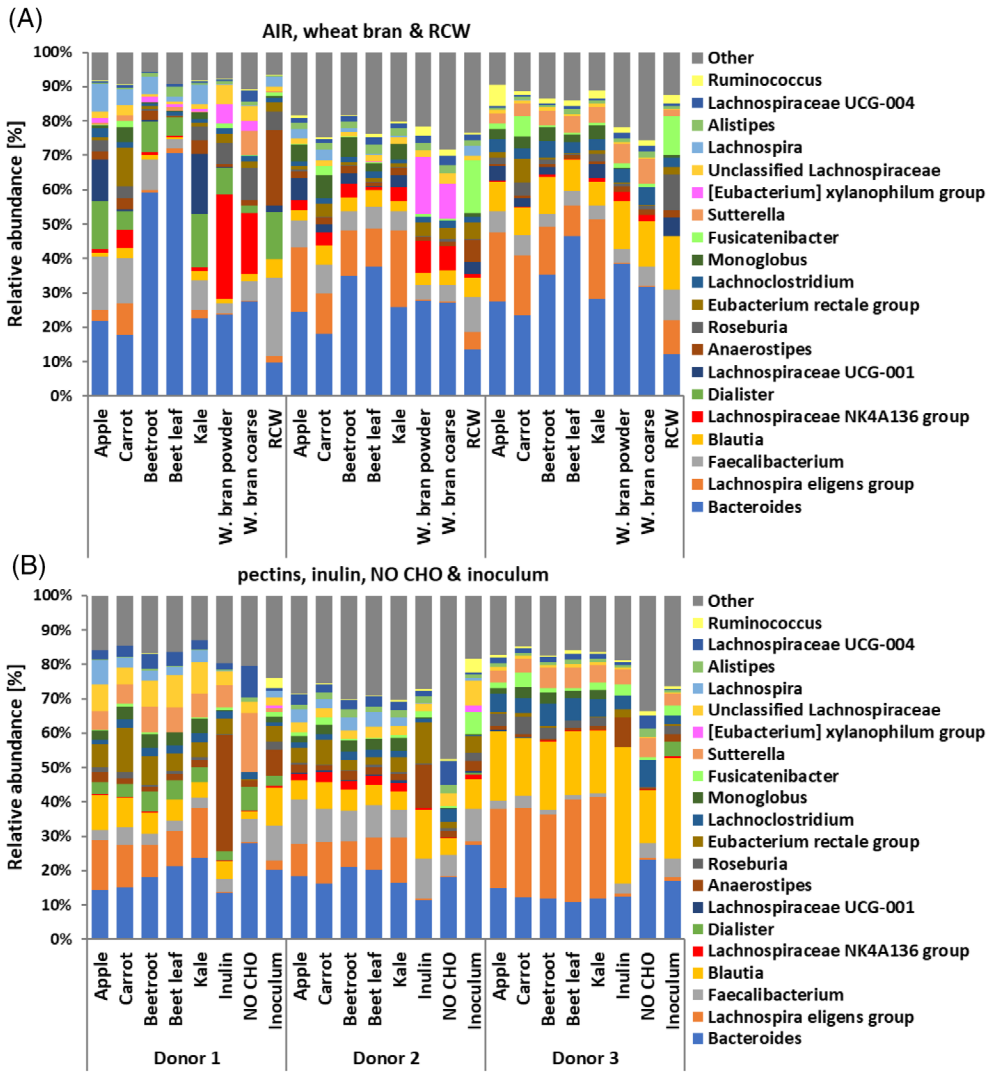

FIGURE 4 Relative abundance of bacterial communities at the genus level after 48 h of incubation (average of three biological replicates). (A) Top 20 genera for alcohol insoluble residue (AIR) and recombined cell wall (RCW). W. Bran, wheat bran. (B) Top 20 genera for pectins, inulin, no carbohydrate control (NO CHO) and faecal inoculum.

communities within a donor. However, compositional differences between the donors on the pectins suggested that faecal donors had a larger influence on community composition than pectin differences. D2 communities had a significantly ($p < 0.001$) higher Shannon diversity and D3 showed less diversity between the substrates than the other donors (Figure S9). Within the D1 communities, a significantly ($p < 0.001$) lower Shannon diversity was observed after incubation on the beet AIRs compared with the other substrates and D2 showed an increased Shannon diversity after incubation on the pectins compared with the AIRs (significant ($p < 0.001$) for AIR from apple, beet leaf, beetroot, kale and wheat bran powder). All the donors displayed higher (but not significant) Shannon diversity on the pectins compared with inulin.

To visualize the effect of donor and substrate on community structure, PCoA based on Bray Curtis dissimilarity of the compositional profiles at the genus level were plotted and statistical differences between the community structures were determined by PERMANOVA. Faecal donor explained 31.3% ($p < 0.001$) of the variation in community composition whereas the different substrates explained 45.8% ($p < 0.001$). The PCoA plots revealed some clear clustering patterns, with D1 and 2 showing some overlap in community structures, in particular after growth on pectins (Figure 5A). Furthermore, the pectins produced more similar communities than the AIRs (Figure 5B), which was confirmed statistically with PERMANOVA by investigating the Bray Curtis Dissimilarity of AIRs and pectins separately. For the pectins, 78.4% ($p < 0.001$) of the variation in the community compositions was explained by donor and only 5.3% ($p < 0.002$) by substrate, whereas for the AIRs and the RCW, it was 34.9% ($p < 0.001$) by donor and 44.0% ($p < 0.001$) by

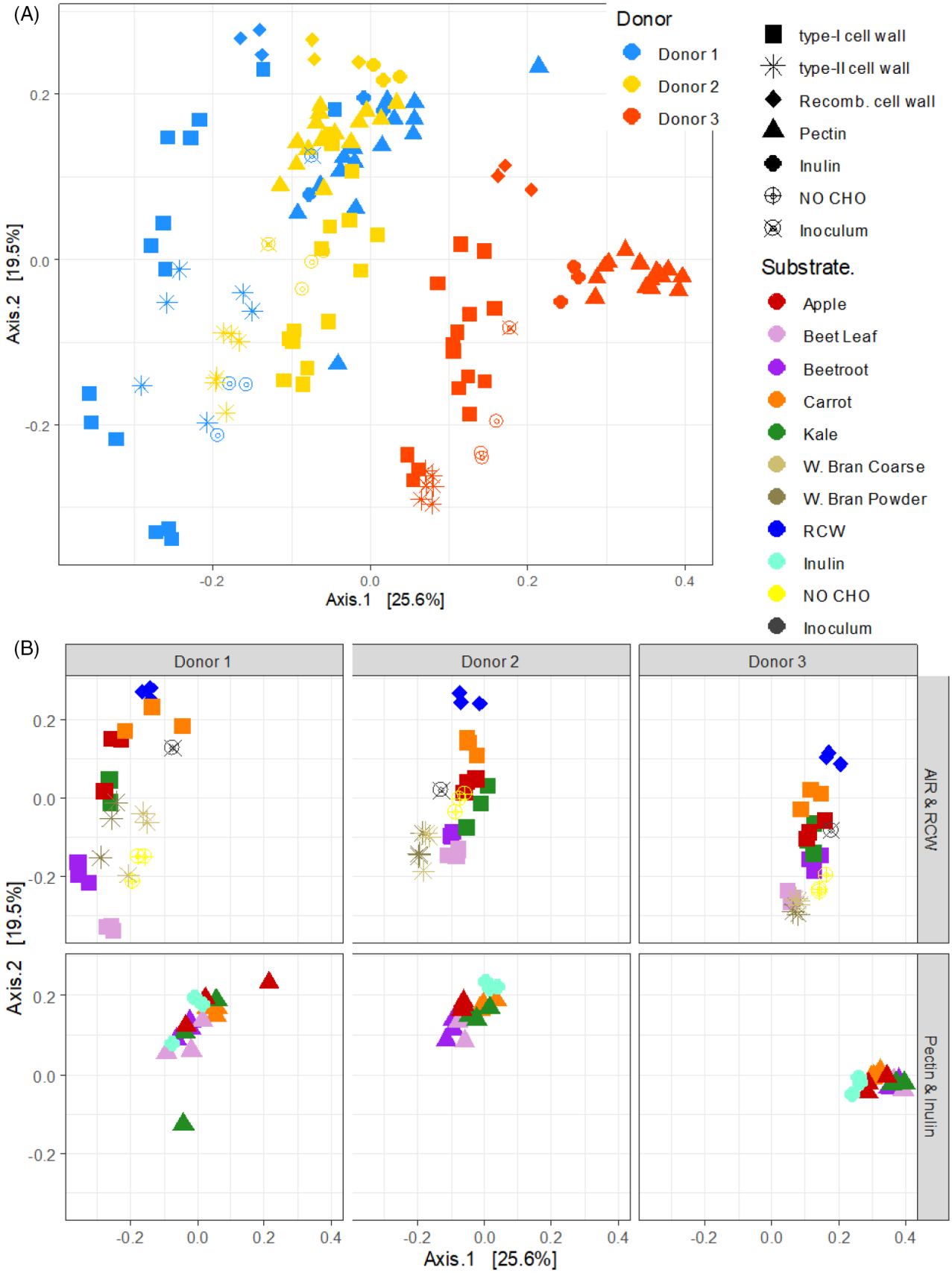

FIGURE 5 PCoA plot showing the community structure after 48 h of incubation as determined by Bray Curtis dissimilarity at the genus level. (A). All donors (labelled by colour) and substrates (labelled by symbol shape) shown together. (B). Individual donors and substrate types (AIR and RCW vs. pectins and inulin) shown separately, with symbol shape indicating substrate type and symbol colour specific substrate.

substrate. Clustering patterns for the AIRs and RCW, on the other hand, were spread along axis 2, with the RCW on top, followed by carrot, apple and kale, while the beets and the wheat brans were closer to the bottom of the plot for all three donors (Figure 5).

Differentially abundant genera after 48 h of incubation

To investigate if there were any differentially abundant genera associated with the different community structures observed between the substrates, LEfSe was conducted between the following incubations: (1) type-I and type-II cell walls, (2) AIRs and RCW (matrix vs no matrix), (3) pectins and AIRs from apple, beet leaf, beetroot, carrot and kale, (4) pectins and inulin and (5) between pectins (Table S4). To accompany the LEfSe analysis and to see how the communities developed compared with the inoculum, the fold change relative to the inoculum was calculated for the differentially abundant genera and all top 20 abundant genera across donors (Table 2) and for each individual donor (Table S5).

LEfSe identified 11 differentially abundant genera between the type-I and type-II cell walls, with type I associated with Lachnospiraceae, including *Lachnospira eligens* group, and the genus *Monoglobus*. The Lachnospiraceae NK4A136 and *[Eubacterium] xylanophilum* groups were the top two differentially abundant genera associated with wheat bran (Tables S4 and 2). For both substrate types there were differences between the donors, which was most pronounced for the *[Eubacterium] xylanophilum* group (Table S5). Higher fold change on the powdered wheat bran compared with the coarse material of the *[Eubacterium] xylanophilum* group for both D1 and D2 indicates the importance of material accessibility. The potential influence of the intact cell wall matrix on bacterial composition was assessed by comparing the RCW substrate to AIR cell wall substrates. LEfSe revealed 13 significant differentially abundant genera. Eight were associated with AIR substrates, with the *Bacteroides* genus showing the highest score, and five with the RCW (Table S4). Of the top three genera associated with the RCW substrate, *Fusicatenibacter* and *Anaerostipes* displayed the largest fold-change differences between RCW and the AIRs (Table 2), with *Fusicatenibacter* mainly responding in D2 and D3 and *Anaerostipes* in D1 and D2 (Table S5).

Comparison of AIRs and pectins from the dicotyledon plants revealed nine significantly differentially abundant genera, all associated with pectin substrates (Table S4) and some resulting in a fold-change increase relative to the inoculum (Table 2). The lack of any differentially abundant genera associated with the AIRs is likely due to the larger variation in community composition across the AIRs. LEfSe analysis of pectin substrates versus inulin revealed three differentially abundant genera on inulin, with a corresponding fold change difference (below 1 for pectins, above 1 for inulin) between the two substrate types for *Anaerostipes* and *Collinsella*. Eight differentially abundant genera were present on pectins, and fold change data reflected the stimulation by pectin but not inulin most clearly for *L. eligens* group, *Lachnospira* and *Monoglobus* (Table 2). The typical bifidogenic effect normally observed with inulin (Chung et al., 2016) was only found for D1. However, the *Bifidobacterium* genus was absent in D3 and showed a higher fold change on the pectins compared with inulin for D2 (Table S5). Pectin has also been reported to stimulate bifidobacteria and our observations are likely due to species-specific differences (Chung et al., 2016). There were no significant differentially abundant bacteria between the different pectins.

When investigating associations with any of the tested type-I AIRs, no genera were found to be significantly differentially abundant when the donor was used as a subclass (i.e. controlling for differences between donors), in line with the donors having relatively large impact on community composition. However, PCoA analysis had revealed similar clustering trends for all donors for the different type-I cell wall substrates (Figure 5B) and LEfSe analysis without donor as a subclass was therefore carried out as it could provide an indication of how the substrates differentially stimulated bacterial growth. This identified a total of 24 significantly differentially abundant genera across the type-I cell wall AIRs, mostly with carrot and beet leaf (Table S6), but fold-change differences between AIRs were mostly quite moderate or several AIRs were found to be high (Table 2), so the results have to be interpreted with caution. Nevertheless, some genera appeared to be stimulated on a specific AIR substrate, such as *Eubacterium rectale* group and *Fusicatenibacter* on carrot AIR and Oscillospiraceae UCG-003 and unclassified Ruminococcaceae on beet leaf. The *Bacteroides* genus was differentially abundant and associated with beet leaf, but also showed a high relative abundance on beetroot, thus the beet-based substrates may stimulate *Bacteroides* more than the other type-I cell wall substrates (Table 2).

Correlations between plant composition, fermentation acids, and differentially abundant bacteria

To get an overview of the potential relationships between substrate composition, differentially abundant bacteria and the fermentation acids, correlations were calculated for the substrate relative composition data against the relative amount of the four main short-chain

TABLE 2 Average fold change relative to the average inoculum across three faecal donors for the top 20 bacterial genera and the genera identified as differentially abundant by LEfSe (Table S4) after 48 h of incubation with different substrates.

		Substrates[a]														Differential abundance[b]				
		AIR								Pectin										
		Matrix																		
		Type-I					Type-II													
Genus	Inoculum	Apple	Carrot	Beetroot	Beet leaf	Kale	Wheat bran powder	Wheat bran coarse	RCW (no matrix)	Apple	Carrot	Beetroot	Beet leaf	Kale	Inulin	Type-I vs type-II	Matrix vs no matrix	Pectin vs type-I AIRs	Pectin vs inulin	Within cell wall type-I
Bacteroides	21.6%	1.1	0.9	2.0	2.4	1.2	1.4	1.3	0.6	0.7	0.7	0.8	0.8	0.8	0.6					
[Lachnospira] eligens group	1.5%	9.3	8.4	6.2	4.7	10.5	0.2	0.1	3.7	10.4	11.2	9.1	10.9	12.6	0.3					
Blautia	16.1%	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.5	0.7	0.7	0.7	0.6	0.6	1.2					
Faecalibacterium	8.4%	1.2	1.1	0.7	0.5	0.7	0.4	0.6	1.6	0.7	0.7	0.5	0.6	0.5	0.7					
Anaerostipes	4.2%	0.4	0.5	0.4	0.2	0.5	0.3	0.3	2.4	0.5	0.3	0.3	0.4	0.4	4.3					
Eubacterium rectale group	3.3%	0.3	2.2	0.2	0.1	0.3	0.7	0.6	1.0	1.1	2.1	1.2	0.8	0.9	1.9					
Sutterella	1.5%	0.7	1.2	0.9	1.3	1.0	1.2	3.1	0.5	2.0	2.1	2.9	2.9	2.7	2.4					
Lachnoclostridium	1.2%	2.1	1.7	1.9	1.7	1.4	2.0	2.3	1.5	2.3	2.2	3.1	3.0	2.5	2.0					
Lachnospiraceae NK4A136 group	0.8%	1.8	3.8	2.4	0.6	2.4	16.7	10.6	0.6	1.0	1.0	1.4	1.2	1.1	0.2					
Unclassified Lachnospiraceae	3.4%	0.4	0.5	0.3	0.3	0.4	0.8	0.8	0.3	1.1	0.8	1.1	1.4	1.2	0.7					
Dialister	0.4%	11.8	4.6	7.5	4.6	13.2	6.5	1.9	11.5	3.0	3.2	5.1	4.7	3.7	2.4					
Monoglobus	0.6%	4.8	7.9	5.4	2.7	5.5	0.1	0.1	1.2	3.3	5.3	5.8	5.0	5.9	0.2					
Subdoligranulum	2.6%	0.3	0.6	0.2	0.2	0.3	0.5	0.6	0.7	1.6	1.2	1.4	1.5	1.2	0.7					
Lachnospiraceae UCG-004	0.5%	1.8	2.0	2.0	2.5	1.8	2.8	5.7	1.9	5.3	4.9	6.1	6.2	4.6	3.2					
Roseburia	2.6%	0.7	0.9	0.3	0.1	0.9	1.0	1.4	2.1	0.7	0.9	0.5	0.4	0.5	0.1					
Lachnospiraceae ND3007 group	0.8%	0.4	4.4	0.4	0.3	1.8	0.6	0.9	1.9	3.1	4.3	2.9	2.6	5.2	2.3					
Lachnospira	0.9%	4.1	2.8	2.3	0.6	2.3	0.1	0.0	2.2	3.9	2.0	2.6	2.5	2.2	0.1					
Fusicatenibacter	2.4%	0.3	1.4	0.1	0.1	0.2	0.5	0.3	3.8	0.4	1.0	0.3	0.2	0.4	0.8					
Lachnospiraceae UCG-001	1.2%	6.3	1.4	1.6	0.1	6.9	0.1	0.0	3.0	0.2	0.2	0.2	0.1	0.1	0.0					
Parabacteroides	0.8%	0.9	1.1	1.0	2.2	1.2	1.4	2.2	0.7	1.9	2.0	2.2	2.4	2.2	1.9					
Alistipes	3.6%	0.2	0.3	0.3	0.7	0.3	0.4	0.5	0.2	0.3	0.3	0.3	0.3	0.3	0.3					
Oscillospiraceae UCG-003	0.2%	1.6	1.7	2.2	5.9	1.6	5.1	10.5	0.8	5.8	4.2	4.5	5.0	3.3	3.8					
Dorea	1.0%	0.7	0.6	1.2	0.7	0.8	0.9	1.4	0.5	0.8	0.6	1.2	1.1	0.9	0.7					
[Eubacterium] hallii group	2.3%	0.7	0.5	0.3	0.1	0.6	0.3	0.3	0.5	0.3	0.2	0.3	0.3	0.3	0.6					
	0.3%	1.7	0.1	1.7	1.0	1.0	25.8	15.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0					

(Continues)

TABLE 2 (Continued)

		Substrates[a]														Differential abundance[b]				
		AIR								Pectin										
		Matrix																		
		Type-I					Type-II													
Genus	Inoculum	Apple	Carrot	Beetroot	Beet leaf	Kale	Wheat bran powder	Wheat bran coarse	RCW (no matrix)	Apple	Carrot	Beetroot	Beet leaf	Kale	Inulin	Type-I vs type-II	Matrix vs no matrix	Pectin vs type-I AIRs	Pectin vs inulin	Within cell wall type-I
[Eubacterium] xylanophilum group																				
Oscillospiraceae UCG-002	0.5%	0.5	0.5	1.1	3.7	0.9	1.7	2.1	0.3	0.8	0.8	1.0	0.9	0.9	0.8					
Collinsella	1.0%	0.2	0.3	0.2	0.2	0.2	0.5	0.6	1.2	0.4	0.3	0.4	0.4	0.3	2.6					
Coprococcus	1.1%	0.3	0.2	0.3	0.2	0.3	0.4	0.6	0.3	0.7	0.6	1.0	0.7	0.5	0.6					
Bifidobacterium	0.9%	0.5	0.3	0.4	0.1	0.2	0.9	0.3	0.6	0.8	0.5	1.2	1.0	0.6	1.2					
[Ruminococcus] gauvreauii group	0.7%	0.5	0.8	0.2	0.2	0.4	0.6	0.5	0.9	0.7	0.8	0.5	0.5	0.5	0.6					
Christensenellaceae R-7 group	1.0%	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.6	0.3					
Erysipelotrichaceae UCG-003	0.0%	0.2	0.3	0.1	0.1	0.2	0.2	0.2	0.8	0.1	0.1	0.1	0.1	0.1	3.5					
Unclassified Ruminococcaceae	0.1%	0.7	0.4	0.9	3.3	0.9	3.1	4.5	0.1	1.4	0.9	0.9	1.2	0.9	1.0					
[Ruminococcus] torques group	0.1%	0.2	0.2	0.3	0.1	0.1	0.9	0.8	0.2	0.2	0.2	0.2	0.2	0.2	0.5					
Butyricicoccus	0.8%	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.3	0.3	0.2	0.2	0.2	0.6					
Odoribacter	0.2%	0.2	0.2	0.2	1.3	0.3	0.2	0.2	0.3	0.3	0.3	0.4	0.3	0.3	0.6					
Bilophila	0.8%	0.1	0.1	0.3	0.9	0.1	0.8	0.2	0.3	0.3	0.6	0.5	0.6	0.7	3.3					
Unclassified Oscillospiraceae	0.2%	0.1	0.1	0.2	0.5	0.2	0.7	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.4					
Oscillospira	0.1%	2.2	2.3	1.4	1.9	1.8	2.7	1.6	0.7	1.2	2.7	1.1	0.7	1.5	6.2					
[Eubacterium] ventriosum group	0.2%	0.4	0.8	0.1	0.2	0.6	0.8	0.7	0.3	0.1	0.2	0.4	0.1	0.1	0.1					
Butyricimonas	0.0%	0.2	0.1	0.1	0.7	0.2	0.5	0.2	0.1	0.2	0.2	0.3	0.2	0.2	0.6					

[a]Conditional formatting indicates high (red) and low (blue) fold change.
[b]Colours indicate if the genus was differentially abundant and associated with the respective substrate/substrate type.

fatty acids formate, acetate, propionate, butyrate and the differentially abundant genera after 48 h of incubation, as well as between the relative SCFA for all substrates (including RCW and Inulin) against the differentially abundant genera (Table S7). *L. eligens* appears to be a highly specialized pectin degrader likely utilizing the homogalacturonan domain, as it correlated strongly with uronic acid for both the type-I cell wall AIRs and pectins, as well as with acetate, which it is known to produce (Lopez-Siles et al., 2012). Lachnospiraceae NK4A136 group and *[Eubacterium] xylanophilum* group are known to include butyrate-producing species (Duncan et al., 2016; Meehan & Beiko, 2014). Both genera were differently abundant and associated with wheat bran and showed a strong correlation with xylose and glucose (originating from arabinoxylan and beta-glucan), both of which also correlated strongly with butyrate (Table S7). After incubation on both wheat bran substrates, the combined relative abundance of Lachnospiraceae NK4A136 group and *[Eubacterium] xylanophilum* group was more than six times higher for D1 and D2 (17.3%–36.2%) than D3 (2.1%–2.7%) (Figure 4). This corresponds well with the higher relative butyrate amounts observed for D1 and D2, compared with D3 on wheat brans (Figure 3), however, this could of course also be due to other bacteria in this complex system.

The genus *Bacteroides* was differently abundant on beet leaf AIR, which was characterized by higher amounts of arabinose and protein (Figure 1), and it correlated with those constituents (Table S7). The *Bacteroides* genus harbours a variety of different species with large substrate flexibility both between and within species (Ndeh & Gilbert, 2018), and 12 species were identified here, with differences in their presence/absence between donors (Table S8). The majority of the *Bacteroides* species generally displayed a higher fold change on the beet samples compared with the other type-I cell wall AIRs, however, the differences were clearest for *B. dorei*, *B. ovatus*, and *B. thetaiotaomicron*. Further strong correlations were observed for other genera (Table S7) that may provide leads for future studies and some are further discussed below.

DISCUSSION

Different approaches are commonly used to quantify the amount of dietary fibre in food, but they do not reveal composition (AOAC method, McCleary et al., 2015) nor do they allow a distinction to be made between glucose originating from cellulose and hemicellulose (Englyst NSP method, Englyst et al., 1994). Here we employed a combination of gravimetric and chemical techniques to obtain a more comprehensive compositional breakdown. While this approach is not the standard method for measuring plant fibre, our results agreed well with available data from the literature for wheat bran (Hollmann & Lindhauer, 2005; Sibakov et al., 2013; Wieser et al., 2020), carrots (Englyst & Cummings, 1984; McCleary et al., 2015) and apple (Dheilly et al., 2016). Compositional data on beetroot and beet leaf were not found in the literature, but data from sugar beet root (belonging to the same species *Beta vulgaris*) also matched our beetroot data relatively well (Zykwinska et al., 2007). Previous data on total dietary fibre in kale also corresponded well with our data (Kahlon et al., 2012). Our data revealed variation both in type and amount of dietary fibre between plants. We found leaves to be the richest sources of cell wall polysaccharides and apple fruit the poorest. As expected, there was compositional overlap between the AIRs of the type-I cell walls, however, there were also differences, with the pectin side-chain composition in beets (rich in arabinose) and carrot (rich in galactose) being particularly striking. When considering fibre from different organs within the same plant, we found that leaves provided the highest amount of total cell wall polysaccharides for both beet and kale. As we only investigated five plant substrates with type-I cell walls, these findings are not generalisable, but they support the importance of consuming a varied diet for achieving an intake of a diverse fibre component.

We assessed the effect of 14 different dietary fibre substrates on bacterial growth, production of fermentation acids and community composition in vitro using faecal samples from three healthy donors. As expected, the more complex substrates gave rise to more variation compared with the corresponding pectins. All pectin-containing substrates stimulated genera with known pectin utilisers, the *L. eligens* group, *Monoglobus*, and *Lachnospira* (Chung et al., 2016; Cornick et al., 1994; Kim et al., 2017). *L. eligens* has been reported to be specialized for pectin degradation, which is in accordance with the constitutive expression of the major PL9 pectin lyase that targets the homogalacturonan domain in *L. eligens* DSM 3376 (Chung et al., 2017). Here, the *L. eligens* group showed a strong correlation with uronic acids and generally a lower increase on the beet samples, which had significantly lower uronic acid amounts compared with the other type-I cell wall AIRs. Therefore, *L. eligens* might be a specialized homogalacturonan utiliser rather than utilizing the other pectin components. These nuances are important if the goal is to use knowledge regarding fibre composition to target specific bacteria. *L. eligens* DSM 3376 is considered to elicit health benefits potentially by inducing the production of the anti-inflammatory cytokine IL-10, as demonstrated in vitro (Chung et al., 2017). Little is known regarding the potential health benefits of the genus *Monoglobus*, as it has only recently been described (Kim et al., 2017). It has previously been demonstrated in faecal incubations that differences in structural features of pectins can affect

bacterial composition (Larsen et al., 2019). Long-chain beet arabinans are strongly associated with cellulose, which can prevent hydrolysis by pectin side chain-degrading enzymes (Zykwinska et al., 2007). This is in accordance with our observation that beet pectins were more difficult to extract than those from other plants. The *Bacteroides* genus, which was stimulated by beet pectins, harbours a variety of different species with large substrate flexibility both between and within species (Ndeh & Gilbert, 2018). It is plausible that they together are able to break down and access the relatively inaccessible beet arabinans. Previous work has shown that both *B. thetaiotaomicron* and *B. ovatus* can utilize arabinan-oligosaccharides, but only *B. thetaiotaomicron* efficiently utilizes intact sugar beet arabinans (Luis et al., 2018). Interestingly, in co-culture, *B. thetaiotaomicron* appears to release arabinan-oligosaccharides that are too large for *B. ovatus* to transport into the periplasm, preventing cross-feeding between the two (Luis et al., 2018). However, in our study, both *B. thetaiotaomicron* and *B. ovatus* displayed higher fold changes on the beet samples compared with the other type-I AIRs, which was also seen for *B. dorei*, suggesting that it may also be involved in cross-feeding of arabinan oligosaccharides. The relatively high abundance of the *Bacteroides* genus on beet leaf was in accordance with significantly higher relative propionate amounts compared with the non-beet AIRs. The other main pectin side chains are galactans, which the carrot material was particularly rich in. Both the carrot AIR and pectin differentially stimulated the *E. rectale* group and the genus *Fusicatenibacter*, which displayed a strong correlation for galactose from both substrates. An analysis of 1321 *E. rectale* genomes showed clear differences between the ability of different strains to utilize certain substrates (Karcher et al., 2020), whereas Sheridan et al. (2016) showed that some *E. rectale* strains could utilize various substrates including fructooligosaccharides, xylooligosaccharides, and galactooligosaccharides. This suggests that *E. rectale* are cross-feeders of polysaccharide breakdown products, which it might be supplied with from species within the *Fusicatenibacter* genus. *Fusicatenibacter saccharivorans* has been shown to harbour α-galactosidase and β-galactosidase enzymes (Takada et al., 2013), but it is not clear if it can utilize intact galactans.

The arabinoxylan-rich wheat bran differentially stimulated the *[Eubacterium] xylanophilum* group, which was in agreement with previous findings using a continuous flow fermenter system incubated with human faecal slurry (Duncan et al., 2016). This genus was absent from the D3 communities, where the other major differentially stimulated genus, Lachnospiraceae NK4A136 group, showed the highest increase. Interestingly, Lu et al. (2021) found *[Eubacterium] xylanophilum* to be enriched by arabinoxylan whereas Lachnospiraceae NK4A136 group was enriched by β-glucan, suggesting that each targets different fractions of wheat bran. Genera representing the known β-glucan degraders *Ruminococcus bicirculans* (Wegmann et al., 2014) and *Coprococcus eutactus* (Alessi et al., 2020) were not found to be enriched here on the wheat bran compared with the AIRs. Duncan et al. (2016) also found *Roseburia* species to be enriched on wheat bran, however, this genus was not found to be differentially abundant here. D1 and D2 communities produced higher relative butyrate amounts from the coarse wheat bran material compared with the powder, suggesting that particle size itself can affect the metabolic output of the community. This reflects previous findings, where faecal batch incubations using wheat bran with a variety of particle sizes showed significantly higher relative butyrate amounts on the coarse material compared with the finer powders (Tuncil et al., 2018). Consequently, the intactness of the food matrix likely affects bacterial growth, as cell wall polysaccharides are less accessible in larger particles. Therefore, the release of the individual polysaccharides from more intact food particles may be more dependent on certain primary degraders (e.g. *R. champanellensis*, Chassard et al., 2012) than if the polysaccharides were present in their purified form. Here we observed clear differences in the community structure between the more intact AIRs compared with the RCW lacking a cell wall matrix. The genus *Bacteroides* was differently abundant and associated with the AIRs, which could be a result of protein fermentation, as the RWC did not have protein. However, as discussed above with the beet samples, it might be that it is the organization of the cell wall matrix that benefits *Bacteroides* species. Together, the *Bacteroides* species can degrade many types of cell wall polysaccharides, which makes them extremely adaptable (Martens et al., 2011).

The butyrate producers *Faecalibacterium* and *Anaerostipes* were differentially abundant and associated with the RCW, which resulted in higher relative butyrate amounts than type-I cell wall AIRs for D1 and D3. A major ecological niche of both genera is likely to be cross-feeders. *Faecalibacterium* species have been shown to grow well on simple carbohydrates but are more limited on complex substrates (Lopez-Siles et al., 2017). *Anaerostipes* species can utilize lactate in addition to carbohydrates (Allen-Vercoe et al., 2012; Duncan et al., 2004; Louis et al., 2022) and it might be that the RCW substrate stimulates certain lactate producers. The major lactate producers (*Bifidobacterium* and *Lactobacillus* species) were not differentially abundant on the RCW, however, many gut bacteria produce lactate as one of their fermentation products, including species within the genus *Fusicatenibacter* (Takada et al., 2013) which was associated with the RCW substrate. *Anaerostipes* was also associated with and stimulated by inulin, which is in agreement with previous observations (Louis et al., 2010; Reichardt et al., 2018).

The differences in community composition within the type-I cell wall substrates were more subtle than other comparisons. However, some interesting differences were observed, such as a stimulation of the *E. rectale* group on carrot AIR, which may be due to the high galactan content of the substrate as discussed above. This suggests that the same fibre component from different plants may indeed stimulate different bacteria in the gut and strengthens the case for the consumption of a varied diet. The results from different plant organs did not reveal a clear pattern and may be of less importance with regard to consuming a diet containing a diverse range of plants, however, this requires further study as only a small selection of plants and plant organs was assessed here.

Here, we used a simple batch culture system that does not ideally reflect the prevailing conditions in the large intestine, however, it enables the parallel analysis of more different substrates than would be possible in complex continuous fermentor systems. We utilized several different measures to assess microbial activity and growth over time and mostly found a relatively good correlation between them. Therefore, total gas production and pH measurements are simple measures that can be used as a proxy for optical density measurements for insoluble substrates. For one donor, the qPCR analysis of the 16S rRNA gene as a marker for growth after 48 h did not agree with the other measures. While we cannot rule out technical issues this seems unlikely, as this was extensively explored. It is possible that bacteria present in some donors may bind to the fibrous material in a way during later stages of incubations that makes it more difficult to recover their DNA during extraction. Furthermore, bacterial lysis may have occurred, as strains with autolytic activity have previously been described (Molinero et al., 2021).

In conclusion, we have demonstrated here that certain compositional features of plants enriched different bacteria during in vitro incubations, suggesting that dietary fibre variety is important for bacterial diversity. In real life, dietary fibre from specific foods is not eaten in isolation as tested here, but consumed as part of a meal. The interplay between dietary fibre and other meal constituents needs to be better understood, as well as food processing and preparation methods that affect fibre structure and physicochemical properties. Faeces from three human donors was analysed here to get an indication of the extend of inter-individual variation in response to different plant fibre preparations. Shared responses as well as differences between donors were detected, showing that inter-individual differences in the microbiota composition of the donors also played an important role in fermentation outcome, which highlights the importance of considering personalized approaches to dietary modulation of the gut microbiota. Altogether, our results suggest that closing the fibre gap using a variety of whole plant foods, or potentially more complex fibre supplements, should promote a diverse microbial ecosystem. Refined prebiotics may also have a role to play in contributing to adequate fibre intake to improve human health, however, there is some evidence that they may elicit detrimental effects at high concentrations under certain circumstances (Singh et al., 2018).

AUTHOR CONTRIBUTIONS

Michael Solvang: Conceptualization (supporting); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); visualization (lead); writing – original draft (equal); writing – review and editing (supporting). **Freda M Farquharson:** Investigation (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (supporting). **Dayan Sanhueza:** Investigation (supporting); methodology (supporting); resources (supporting); writing – review and editing (supporting). **Graham Horgan:** Formal analysis (supporting); writing – review and editing (supporting). **Wendy R. Russell:** Conceptualization (supporting); funding acquisition (supporting); methodology (supporting); supervision (supporting). **Petra Louis:** Conceptualization (lead); data curation (supporting); funding acquisition (lead); methodology (equal); project administration (lead); supervision (lead); visualization (supporting); writing – original draft (equal); writing – review and editing (lead).

ACKNOWLEDGEMENTS

Michael Solvang was funded by a Scottish Government Rural and Environment Science & Analytical Services (RESAS) PhD studentship and Freda Farquharson, Graham Horgan, Wendy Russell and Petra Louis also received financial support from RESAS. We would like to thank Sylvia Duncan for the gift of the wheat bran material, Donna Henderson at the Analytical Services Division of the Rowett Institute for carrying out part of the compositional analysis of substrates and short-chain fatty acid analysis, the Centre for Genome Enabled Biology and Medicine at the University of Aberdeen for 16S rRNA amplicon sequencing, Sophie Shaw for help and advice on bioinformatics analysis and Stephen Fry for training in carbohydrate analysis. The authors would like to acknowledge the support of the Maxwell Compute Cluster funded by the University of Aberdeen.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The processed data that supports the findings of this study are available in the supplementary material of this article, raw data not provided are available from the corresponding author upon reasonable request. Sequence

data have been deposited in the National Centre for Biotechnology Information sequence read archive (NCBI SRA) under accession numbers PRJEB48569.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Solvang, M., Farquharson, F.M., Sanhueza, D., Horgan, G., Russell, W.R. & Louis, P. (2023) Beyond purified dietary fibre supplements: Compositional variation between cell wall fibre from different plants influences human faecal microbiota activity and growth in vitro. *Environmental Microbiology*, 1–21. Available from: https://doi.org/10.1111/1462-2920.16368