Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon

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Abstract

Dietary plant cell wall carbohydrates are important in modulating the composition and metabolism of the complex gut microbiota, which can impact on health. Pectin is a major component of plant cell walls. Based on studies in model systems and available bacterial isolates and genomes, the capacity to utilize pectins for growth is widespread among colonic Bacteroidetes but relatively uncommon among Firmicutes. One Firmicutes species promoted by pectin is *Eubacterium eligens. E. eligens* DSM3376 utilizes apple pectin and encodes a broad repertoire of pectinolytic enzymes, including a highly abundant pectate lyase of around 200 kDa that is expressed constitutively. We confirmed that certain *Faecalibacterium prausnitzii* strains possess some ability to utilize apple pectin and report here that *F. prausnitzii* strains in common with *E. eligens*, can utilize the galacturonide oligosaccharides DP4 and DP5 derived from sugar beet pectin. *F. prausnitzii* strains have been shown previously to exert anti-inflammatory effects on host cells, but we show here for the first time that *E. eligens* strongly promotes the production of the anti-inflammatory cytokine IL-10 in *in vitro* cell-based assays. These findings suggest the potential to explore further the prebiotic potential of pectin and its derivatives to re-balance the microbiota towards an anti-inflammatory profile.

Introduction

The dense and complex microbial communities that colonise the human colon are largely supported by dietary substrates that escape digestion by host enzymes. The colonic microbiome encodes a far wider range of biochemical functions than the host genome (Kaoutari *et al.*, 2013) in particular with respect to enzymes capable of degrading complex carbohydrates that allow them to take advantage of these dietary components as energy sources (Flint *et al.*, 2012). Diet composition and especially consumption of non-digestible polysaccharides is likely to have a major impact in driving the species composition of gut

microbial communities and their metabolic outputs (Duncan *et al.*, 2007; Walker *et al.*, 2011; David *et al.*, 2014). This can have a substantial impact on gut health and more generally on systemic health (Louis *et al.*, 2004; Russell *et al.*, 2011) with the impact of gut microorganisms on inflammation having an important role (Palm *et al.*, 2015). Analysis of 16S rRNA gene sequences shows that the healthy human colon hosts two bacterial phyla, the Firmicutes and Bacteroidetes, which usually make up more than 75 % of the total microbiota (Hold *et al.*, 2002; Eckburg *et al.*, 2005; Duncan *et al.*, 2007; Huttenhower *et al.*, 2012).

Plant cell walls are a valuable source of carbon for gut bacteria and are primarily composed of pectins, cellulose and hemicellulose. Pectins are structurally highly complex and are classified into the following three components, homo-polygalacturonan (PG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (Caffall & Mohnen, 2009). Homogalacturonan is the most abundant pectic polymer and has the simplest structure being comprised of linear chains of α 1,4 D-galacturonate residues. The carboxylic groups of D-galacturonate are methyl esterified to various degrees (usually up to around 70-80 %) and these residues may also be acetylated. In contrast, polygalacturonate, RG I and RG II, are highly branched polymers. The RG I backbone consists of L-rhamnose and D-galacturonate residues and is substituted by side chains of galactan, arabinan and arabinogalactan whilst RG II contains a short galacturonan backbone with various sugar side chains that tend to occur in groups (O'Neill *et al.*, 2004). Pectin degradation involves glycoside hydrolases (GH), polysaccharide lyases (PL) and carbohydrate esterases (CE) (Flint *et al.*, 2012). Lyases act only on acidic polymers such as PG and RG I, with five known families of pectate lyases (Hugouvieux-Cotte-Pattat *et al.*, 2014) and two of rhamnogalacturonate lyases. Methyl esterases, acetyl esterases or feruloyl esterases act as accessory enzymes that remove substituent groups, thus facilitating the action of the depolymerases (Fries *et al.*, 2007).

Pectin degradation is a common trait amongst Gram-negative *Bacteroides* species found in the human colon and the gene clusters (PULs) responsible for utilization of different components of this complex material have been identified and investigated in detail for several species (Sheridan *et al*., 2016). In contrast, relatively few Gram-positive bacterial species have been reported to ferment pectin or pectin breakdown products (Salyers *et al.*, 1977; Lopez-Siles *et al.*, 2012; Chung *et al.*, 2016). The possibility that these Firmicutes species may however include species that could confer anti-inflammatory activity and deliver health benefits (Sokol *et al.*, 2008; Martín *et al.*, 2014; Miquel *et al.*, 2015; Rossi *et al.*, 2015) makes it important to investigate them further. We report here that *Eubacterium eligens* is a specialist pectin-degrading Firmicutes species that has the potential to deliver anti-inflammatory activity by promoting the production of IL-10 by epithelial cells. We also show that a second abundant Firmicutes species that promotes anti-inflammatory activity, *F. prausnitzii*, while unable to degrade pectin also shares with *E. eligens* the ability to utilize defined pectic oligosaccharides for growth.

Materials and Methods

Homogalacturonan oligomer (DP4 and DP5) preparation

Homogalacturonan oligomers (DP4 and DP5) were prepared as described previously (Holck *et al.*, 2011). Briefly, 3 % (w/w) sugar beet pectin from Nordic Sugar (Nakskov, Denmark) in 50 mM Tris buffer (pH 8) was treated with 0.5 % pectin lyase and filtered. The filtrate was subjected to alkaline de-esterification to remove methoxyl and acetyl groups. The homogalacturonan oligomers were separated by ion exchange chromatography and the identity of each peak and purity was verified by HPAEC-PAD analysis using a CarboPac PA1 analytical column (Dionex Corp., Sunnyvale, CA, USA). The elution of the unsaturated oligosaccharides was monitored by UV absorption at 235 nm. Elution was performed at a flow rate of 20 mL/min with water and ammonium formate as eluents. Before injection of the sample, the column was equilibrated with 20 mM ammonium formate for two column volumes (CV). After injection, the column was washed with 20 mM ammonium formate for three CV, followed by elution with a gradient from 20 mM to 430 mM ammonium formate for 17.5 CV. After elution, the column was regenerated with 1 M ammonium formate for three CV and with water for three CV. The relevant elution fractions were collected and lyophilized five-six times to obtain a dry sample with a residual ammonium formate concentration of less than 1% (w/w) as determined by a formic acid assay kit from Megazyme (Wicklow, Ireland).

The identity of each peak and purity of the dry samples were verified by HPAEC-PAD analysis using a BioLC system equipped with a CarboPac PA1 guard column and a CarboPac PA1 analytical column (Dionex Corp., CA, USA). Isocratic elution took place with 500 mM sodium acetate and 50 mM NaOH at a flow rate of 1 mL/min for 25 minutes. Previously purified homogalacturonan oligomers identified by MALDI-MS and MS/MS as described by Holck *et al.* (2011) were used as standards. The identity of the peaks was confirmed by

MALDI-TOF analysis with an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Germany). The average yields per gram dry matter pectin for DP4 and DP5 were 45 mg/g and 37mg/g respectively.

Preparation of bacterial cultures for carbohydrate utilization, assessment of bacterial growth using a miniaturised microtitre plate assay and for anti-inflammatory cytokine analysis

Six bacterial strains were tested for their ability to utilise apple pectin (Sigma Aldrich, Poole, Dorset, UK) and highly purified DP4 and DP5 (Holck *et al.*, 2011) substrates using the microtitre plate method in triplicate (technical replicates) as described previously (Scott *et al.*, 2014). Bacterial strains included four strains, that represented the two phylogroups of *F. prausnitzii* (A2-165, S3L/3, L2-6, M21/2) (Lopez-Siles *et al.*, 2012) held by the Rowett Institute, Aberdeen, *Eubacterium eligens* (DSM3376)= ATCC27750 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany and *Bacteroides thetaiotaomicron* (ATCC 5482) was from the American type culture collection (ATCC), Manassas, VA, USA. Cultures for growth studies were pre-prepared by culturing on M2 medium containing 0.2% (w/v) of each glucose, cellobiose and soluble potato starch (Miyazaki *et al.*, 1997) for 20-24 h at 37 °C under CO_2. Briefly, sterile microtitre plates were maintained in an anaerobic cabinet (Don Whitley, Shipley, UK) for 24 h prior to adding 200 μL anaerobic pre-reduced basal M2 medium containing either 0.2 % (w/v) of glucose, apple pectin, DP4 or DP5 as the single growth substrate. The wells were then inoculated with overnight cultures grown anaerobically in M2GSC medium in triplicate for each substrate or basal medium as control. The plates were covered with Q-optical seals (Bio-Rad, UK) and then the plates were incubated for 24 h at 37 °C in a Tecan Safire 2 microplate reader (Tecan Group Ltd, Switzerland) with optical readings at 650 nm taken

every hour following low speed shaking for 5 s, as described previously (Scott *et al.*, 2014). The resazurin dye indicated that this was sufficient to maintain anaerobiosis.

To test *E. eligens* DSM3376 and *F. prausnitzii* A2-165 for cytokine profiles (see below), the commensal bacteria were grown in YCFA medium (Lopez-Siles *et al.*, 2012) containing 0.2 % glucose and modified to contain only 10 mM acetate and no other short chain fatty acids (SCFA). The cultures were grown to late exponential phase (OD_{650} 0.7-0.8). *Lactobacillus plantarum* WCFS1 was isolated from human saliva (Rossi *et al.*, 2016). *L. plantarum* WCFS1 was cultured overnight to stationary phase in MRS broth (Merck, Germany). All bacteria were harvested and washed in PBS as described previously (Rossi *et al.*, 2016).

Short chain fatty acid (SCFA) analysis

SCFA formation was measured in culture supernatants by gas chromatography as described previously (Richardson *et al.*, 1989). Briefly, following derivatisation of the samples using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide, the samples were analysed using a Hewlett Packard gas chromatograph fitted with a fused silica capillary column with helium as the carrier gas. The lower detection limit was 0.2 mM.

Proteomic analysis

Proteomic analysis was performed on cell pellets obtained from triplicate biological experiments. *E. eligens* DSM3376 was grown anaerobically in 800 mL of modified YCFA medium (Lopez-Siles *et al.*, 2012) , modified by inclusion of 1 % (v/v) rumen fluid and addition of 1 mM butyric acid in the SCFA mix, supplemented with 0.5 % (w/v) glucose or apple pectin (Sigma Aldrich, Poole, Dorset, UK) at 37 °C to an OD_{650} between 0.7 and 0.8.

After centrifugation at 10,000 RPM, at 4 °C for 30 min, cell pellet were then washed in sterile phosphate buffered saline (PBS) three times and re-suspended with Rabilloud buffer (7 M urea, 2M Thio-urea 4% CHAPS, 0.5 % Biolite Ampholite; pH 3–10) for protein extraction. Protein concentrations of the supernatant and cellular fractions were measured using the RCDC assay following the steps describe in the manual (Lowry et al., 1951). Aliquots of 100 mg of protein were precipitated in 25 % trichloroacetic acid (TCA)/20 mM dithiothreitol (DTT) for 1 h on ice, followed by centrifugation at 10,000 g for 10 min at 4ºC. Pellets were washed four times with 1 mL ice-cold acetone containing 20 mM DTT. After removing the acetone, the protein pellets were re-suspended in Rabilloud buffer and 200-250 µg of protein separated on two dimensional gels using 17 cm IPG strips, pH 4-7 as described elsewhere (Vodovnik et al., 2013). Gel images were analysed with PD Quest Advanced 8.0.1 (Bio-Rad, Hertforshire, UK). The densest spots were excised from the gels manually, trypsinized and subjected to protein identification by Nano LC MS/MS as described elsewhere (Vodovnik et al., 2013), followed by analysis of the total current ion data using the MASCOT search engine as described previously (Vodovnik et al., 2013).

Human peripheral blood mononuclear cell (PBMC) isolation and stimulation
Peripheral blood of three healthy blood donors was obtained from the Sanquin Blood bank in Nijmegen (The Netherlands). A written informed consent was obtained from each volunteer before sample collection. Buffy coats containing the concentrated leukocyte suspension were obtained as described previously (Van Hemert et al., 2010), washed and re-suspended in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Breda, The Netherlands) to a final concentration of 1×10^6 cells/mL. Approximately 5 x 10^5 cells were added to each well of a 48-well tissue culture plate and incubated overnight at 37 °C in an incubator containing air and 5 % CO_2.

For the PBMC stimulation experiments, the different bacteria at a cell to bacteria ratio of approximately 1:5 or bacterial culture supernatant (2 % v/v) were added for 24 h. IMDM containing the same mixtures and concentrations of SCFA or lactic acid produced during *in vitro* culture of the different bacteria were added at the same volume as the culture supernatant to control for the effects of SCFA and lactate. The PBMCs were stimulated at 37 °C and 5 % CO_2 for 24 h. After incubation, the PBMC culture supernatants were collected and frozen at -20°C until cytokines were measured.

Cytokine measurement

Cytokines IL-12p70, IFN-γ, TNF-α, IL-1β, and IL-10 produced in PBMC culture supernatants were measured by flow cytometry using the FACS CantoII (Becton Dickinson, Oxford, UK) and the Cytometric Bead Array Flexset (BD Biosciences, Breda, the Netherlands) per the manufacturer's instructions and the data was analyzed using the BD FCAP software. Significance was tested using one-way ANOVA.

Results

Distribution of pectin-degrading among human colonic Firmicutes. Figure 1 shows the distribution of potential pectin-degrading enzymes in a selection of 20 dominant species (including four Bacteroidetes and 14 Firmicutes) of human colonic anaerobes for which genome analysis is available via the carbohydrate active enzyme (CAZyme) classification system (www.cazy.org). It can be seen that all four *Bacteroides* species examined carry representatives of carbohydrate esterase families (CE8 and CE12) implicated in the removal of methyl and acetyl groups from pectin, together with glycoside hydrolases and polysaccharide lyases implicated in polygalacturonan, rhamnogalacturonan and pectin

degradation (GH28, GH78, GH105, GH106, PL1, PL9, PL10, PL11, PL22). Among the

Firmicutes, potential pectin-degrading genes are present in some species, but generally in

smaller numbers and representing a less balanced repertoire than that seen in the *Bacteroides*

species (Fig. 1). *E. eligens* ATCC27750=DSM3376, however, possesses two glycosyl

hydrolases (GH28 and GH105), two pectate lyase families (PL1 and PL9) and the two

families of carbohydrate esterases (CE1 and CE12) involved with pectin utilization (Fig. 1).

A few other Firmicutes species, possess more limited gene sets potentially concerned with

pectin-degradation; these include *Roseburia* spp. that carry GH28, GH78 and GH105 genes,

R. champanellensis that encodes multiple polysaccharide lyases (Moraïs *et al.*, 2016) and

some *F. prausnitzii* strains. (Fig. 1). Significantly, a recent study by Chung *et al.* (2016)

whereby apple pectin was supplied as sole carbohydrate substrate to human colonic microbial

communities in anaerobic continuous culture and used 16S rRNA amplicon sequencing to

detect the most successful competitors for this substrate demonstrated stimulation of six

different *Bacteroides* spp., among the Firmicutes only *Eubacterium eligens* was strongly

promoted. Analysis of these data revealed that *E. eligens* increased to an average of 15 % of

total 16S rRNA gene sequences in the pectin-fed communities compared with only 1 % in the

faecal inoculum (Fig. 2).

Growth of *Eubacterium eligens* and *Faecalibacterium prausnitzii* strains on pectin and

pectic oligosaccharides. *F. prausnitzii* strains showed variable growth with apple pectin as

the added carbon source (Fig. 3; Table S1). Although Sigma apple pectin contains around 4

% contaminating starch (Despres *et al.*, 2016), *F. prausnitzii* does not grow on starch (Lopez-

Siles *et al.*, 2012) suggesting that it may be able to utilize pectic oligosaccharides. We

therefore investigated the ability of strains compared to *F. prausnitzii* A2-165 and *B.*

thetaiotaomicron 5482 to utilize two galacturonide oligosaccharides recently purified from

sugar beet pectin, DP4 and DP5 (Holck *et al.*, 2011). *E. eligens* DSM3376 and *B.*

thetaiotaomicron 5482 showed rapid growth both on DP4 and DP5. *F. prausnitzii* A2-165 could utilize DP4, but not DP5, while *F. prausnitzii* L2-6 showed weak growth on both oligosaccharides (Fig. 3). *F. prausnitzii* S3L/3 grew better on DP5 than on DP4, while *F. prausnitzii* M21/2 showed no growth with either substrate (Fig. 3). This suggests that the limited growth of some *F. prausnitzii* strains on pectins is explained by the ability to utilize certain pectin oligosaccharides as growth substrates.

***E. eligens* proteins expressed during growth with glucose or pectin**. Cell-associated proteins and culture supernatants were obtained from cultures of *E. eligens* DSM3376 grown with 0.5 % apple pectin or 0.5 % glucose as energy sources (see Methods). Analysis of 18 proteins that showed greater expression on pectin than on glucose revealed three (uronate isomerase, 2-dehydro-3-deoxyphosphogluconate aldolase, gluconate 5 dehydrogenase) that are likely to be involved in the metabolism of galacturonic acid (Servinsky *et al.*, 2014) but did not include any of the 14 hydrolases, esterases or lyases predicted from the genome to be concerned with pectin depolymerization. When we analyzed the 10 most abundant proteins of MW >50kDa that were constitutively expressed during growth in both substrates, these were found to include a large family 9 pectin lyase of 1731 amino acids that carries a likely C-terminal cell wall anchor (Fig. S1). Proteins that were more highly expressed on glucose than on pectin included flagellin.

Cytokine profiling of human peripheral mononuclear cells (PBMC) stimulated with to *E. eligens* and *F. prausnitzii*. This work suggests that there might be potential for using pectin and its derivatives in prebiotic approaches to promote *E. eligens* and possibly *F. prausnitzii* within the colonic microbiota. Several reports have demonstrated an anti-inflammatory activity of *F. prausnitzii in vitro* and *in vivo* (Sokol *et al.*, 2008; Rossi *et al.*, 2016), but nothing is known about the immunomodulatory effects of *E. eligens*. Here, stimulation of peripheral blood mononuclear cells with *E. eligens* DSM3376 induced

secretion of high amounts of IL-10 (8867 pg/mL) but IL-12 was below the detection limit. The amount of IL-10 produced by PBMCs stimulated with *E. eligens* was greater than that measured after stimulation with *F. prausnitzii* A2-165 which has previously been shown to have a high capacity to induce IL-10 in human and mouse immune cells (Sokol *et al.*, 2008; Rossi *et al.*, 2016). *E. eligens* induced only low amounts of IFN-γ and IL-1β compared to *F. prausnitzii* but greater amounts of TNFα (2234 pg/mL) (Fig. 4). Furthermore, PBMCs stimulated with the culture supernatants of *E. eligens* induced significant production of IL-10 (2554 pg/mL), but very small amounts of all the other cytokines including IL-12p70 which was below the detection limit (5 pg/mL) (Fig. 4).

Following growth, the fermentation acids formed by these strains were determined by GC analysis (see Materials and Methods). *E. eligens* formed 7.5 mM formate, 15.9 mM acetate and 0.8 mM lactate and *F. prausnitzii* A2-165 formed 5.4 mM formate and 10.1 mM butyrate. Addition of 2 % v/v of the mixture of SCFA and lactic acid measured in the bacterial culture supernatants did not induce cytokine production in PBMCs. To rule out the possibility that the SCFAs and lactic acid produced during *in vitro* culture of *E. eligens* was responsible for the high production of IL-10 in PBMCs we added cell culture medium containing the same acid mixture to PBMCs with or without *L. plantarum* as a stimulus. The *E. eligens* acid mixture alone did not elicit any cytokines in PBMCs and had no effect on the amounts of cytokines induced by *L. plantarum* indicating they were not modulating the IL-10 response upon immune activation (Fig. S2).

Discussion

Early culture-based studies suggested that while pectin utilization was widespread among human colonic *Bacteroides* species, this capacity was limited among the Firmicutes (Salyers

et al., 1977). This suggestion has recently been confirmed by studies using continuous culture, along with high throughput sequence-based analysis of microbiota composition, to detect species stimulated within the colonic microbiota when apple pectin is provided as sole carbon and energy source (Chung *et al.*, 2016). This selectivity of pectin as a growth substrate corresponds to a limited distribution of gene families implicated in pectin utilization within genomes from human colonic Firmicutes. One Firmicutes species that is reported to utilize pectin (Salyers *et al.*, 1977; Lopez-Siles *et al.*, 2012) and that was strongly stimulated by pectin in continuous cultures (Salyers *et al.*, 1977; Lopez-Siles *et al.*, 2012; Chung *et al.*, 2016) however is *E. eligens*. We show here that this species possesses a repertoire of pectin utilization genes belonging to at least six different CAZyme families. Interestingly, the major PL9 pectin lyase in *E. eligens* DSM3376 was found to be produced constitutively and hydrolases and lyases predicted to act on pectin were not detected among proteins showing induced expression on pectin, although enzymes involved in galacturonic acid metabolism were induced on pectin. Another highly specialised Firmicutes species within the colonic microbiota, the starch-degrader *Ruminococcus bromii*, was recently shown to produce its amylolytic system constitutively (Ze *et al.*, 2015).

The selectivity of pectin as a growth substrate among the Firmicutes and Bacteroidetes (Chung *et al.*, 2016) suggests that pectin or pectin derivatives could be of considerable interest as a basis for prebiotics. This depends critically however on knowing the likely impact upon the host of bacterial species that may be promoted by pectin. For example, *F. prausnitzii* strains HTF-F and A2-165 have previously been reported to attenuate development of colitis in different mouse models (Sokol *et al.*, 2008; Martín *et al.*, 2014; Rossi *et al.*, 2015) and have a high capacity to induce IL-10 in murine and human dendritic cells with little or no IL-12 secretion (Rossi *et al.*, 2016). Moreover, supernatants of strain A2-165 were also shown to attenuate cytokine-mediated NF-kB activation in human Caco-2

intestinal epithelial cells (Sokol *et al.*, 2008). In an *in vivo* mouse model of nasal tolerance to ovalbumin, *F. prausnitzii* A2-165 enhanced ovalbumin-specific T cell proliferation and reduced the proportion of IFN-γ+ T cells in CLNs (Rossi *et al.*, 2016). Similarly, *in vitro F. prausnitzii* A2-165 stimulated BMDCs increased ovalbumin-specific T cell proliferation and reduced the number of IFN-γ+ T cells. These mechanisms may contribute to the anti-inflammatory effects of *F. prausnitzii* in colitis and support the notion that this abundant bacterium might contribute to immune homeostasis in the intestine via its anti-inflammatory properties.

To our knowledge, no previous work has been performed on the interactions of host cells with *E. eligens*. Here we show that *E. eligens* DSM3376 induced relatively large amounts (approximately 9000 pg/mL) of the anti-inflammatory cytokine IL-10 and low amounts of IL-1β and IFNγ in human PBMCs compared to *F. prausnitzii*. Furthermore, IL-10 was the only cytokine induced by the *in vitro* culture supernatant of *E. eligens*. This effect was not due to the mix of SCFA and lactic acid produced by *E. eligens in vitro* as the same mixture of acids did not induce cytokine production by PBMCs or modulate the cytokine profile induced by stimulation with *L. plantarum*.

In contrast to *F. prausnitzii*, *E. eligens* possesses genes that encode flagella and we detected flagellar protein expression here in the proteome of both glucose and pectin grown cells, confirming expression of the flagellar genes found in this species (Servinsky *et al.*, 2014). Flagellins associated with pathogenic bacteria are pro-inflammatory during infection, but there is also some evidence that the TLR5 signalling by flagellins of commensal bacteria via TLR5 may suppress inflammation *in vivo* (Vijay-Kumar *et al.*, 2010) and the impact of flagellins expressed by commensal gut anaerobes, which also include *Roseburia* spp., *Eubacterium rectale* and *E. siraeum* (Neville *et al.*, 2013), remains unclear. Interactions of

Gram-negative *Bacteroides* spp. with the host have been studied in greater detail and it is known that human colonic *Bacteroides* spp. can exert a range of effects that range from opportunistic pathogenesis to anti-inflammatory action (Telesford *et al.*, 2015). The net outcome will therefore depend both on the characteristics of individual species that become enriched by pectin in an individual's microbiota and on their collective effects as part of a pectin-enriched microbial community and previous studies suggest that *E. eligens* would compete well for pectin (Chung *et al.*, 2016).

Pectin represents a structurally highly complex and chemically heterogeneous family of molecules. This offers the prospect of identifying components, for example partial degradation products, that might provide different prebiotic specificities and potential by comparison with native plant pectins. For example, we found here that some *F. prausnitzii* strains, although limited in its ability to utilize pectin, shared with *E. eligens* the ability to grow on purified pectic-oligosaccharides DP4 and DP5. This suggests that such compounds might offer a successful prebiotic approach for promoting these potentially beneficial Firmicutes species. On the other hand we must assume that similar selection of pectin-utilizing species would also occur among the Bacteroidetes, making it desirable to understand better the consequences of promoting different species within this genus.

In conclusion, our findings show that the Firmicutes species *E. eligens* is an important, specialist degrader of diet-derived pectins in the human colon that produces a constitutive pectate lyase. This bacterium and its culture supernatant strongly stimulated production of anti-inflammatory IL-10 in PBMCs, suggesting that it may have potent anti-inflammatory effects *in vivo* and therefore should be given serious consideration as a promising next generation probiotic.

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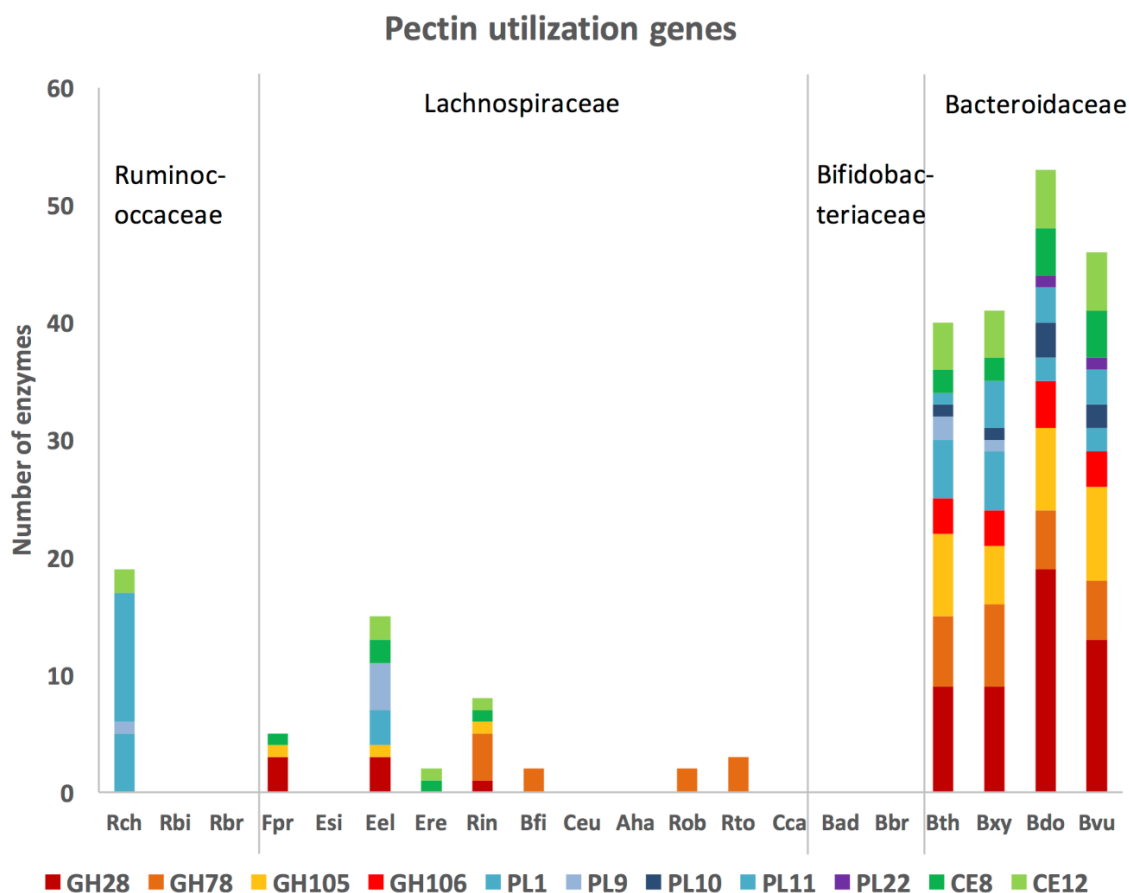
Figure legends

Figure 1. Predicted distribution of enzymes concerned with pectin degradation. Genome analysis derived obtained from the Carbohydrate-Active enZYmes database (CAZY) on enzymes associated with pectin degradation: Glycoside Hydrolase (GH family 28, 78, 105, 106), Polysaccharide Lyase (PL family 1, 9, 10, 11, 22) and Carbohydrate Esterase (CE family 8, 12) for bacterial species from the Ruminococcaceae, Lachnospiraceae, Bifidobacteriaceae and Bacteroidaceae. Rch = *R. champanellensis* (18P13), Rbi = *R. bicirculans* (80/3), Rbr = *R.bromii* (L2-63), Fpr = *F. prausnitzii* (S3L/3), Esi = *E. siraeum* (70/3), Eel = *E. eligens* (ATCC27750), Ere = *E. rectale* (M104/1), Rin = *R. intestinalis* (XB6B4), Bfi= *B. fibrisolvens* (16/4), Ceu = *C. eutactus* (ART55/1), Aha = *A. hadrus* (SSC/2), Rob = *R. obeum* (A2-162), Rto = *R. torques* (L2-14), Cca = *C. catus* (GD/7), Bad = *B. adolescentis* (ATCC15703), Bbr = *B. breve* (UC2003), Bth = *B. thetaiotaomicron* (VPI5482), Bxy = *B. xylanisolvens* (XB1A), Bdo = *B. dorei* (HS1_L1_B_010), Bvu = *B. vulgatus* (ATCC8482).
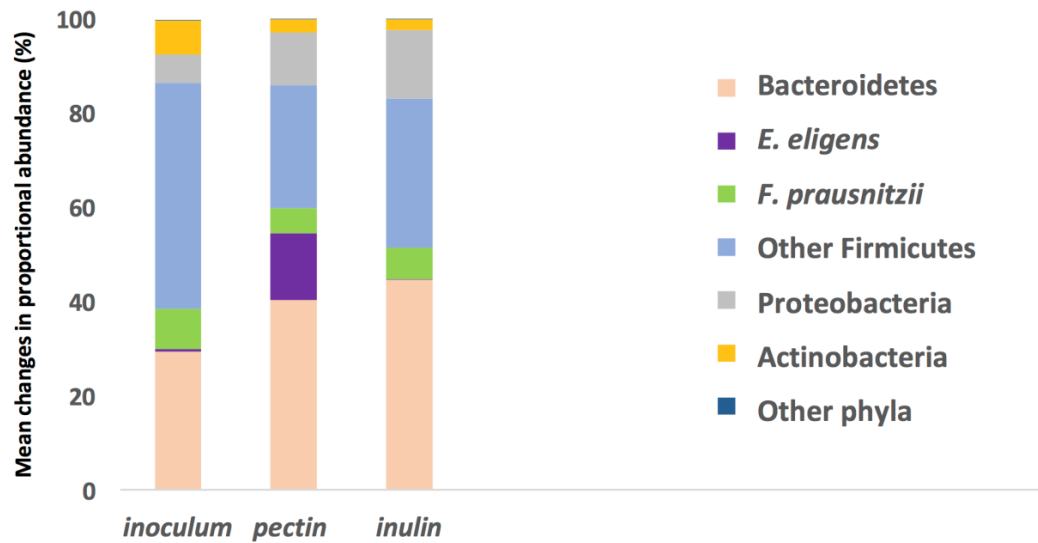
Figure 2. Enrichment of *Eubacterium eligens* during continuous culture of human colonic microbial communities supplied with apple pectin as growth substrate. Mean relative abundance of two Firmicutes species, *Eubacterium eligens* and *Faecalibacterium prausnitzii* and of the dominant bacterial phyla present, based on sequencing of 16S rRNA gene amplicons from the study of Chung et al (2016). Microbiota composition is shown for the faecal inocula, and between days 3-12 of incubation post-inoculation with either apple pectin or inulin as the sole carbohydrate sources in anaerobic continuous culture. Means refer to three sets of experiments (two vessels per experiment) involving different faecal donors (Chung et al, 2016).
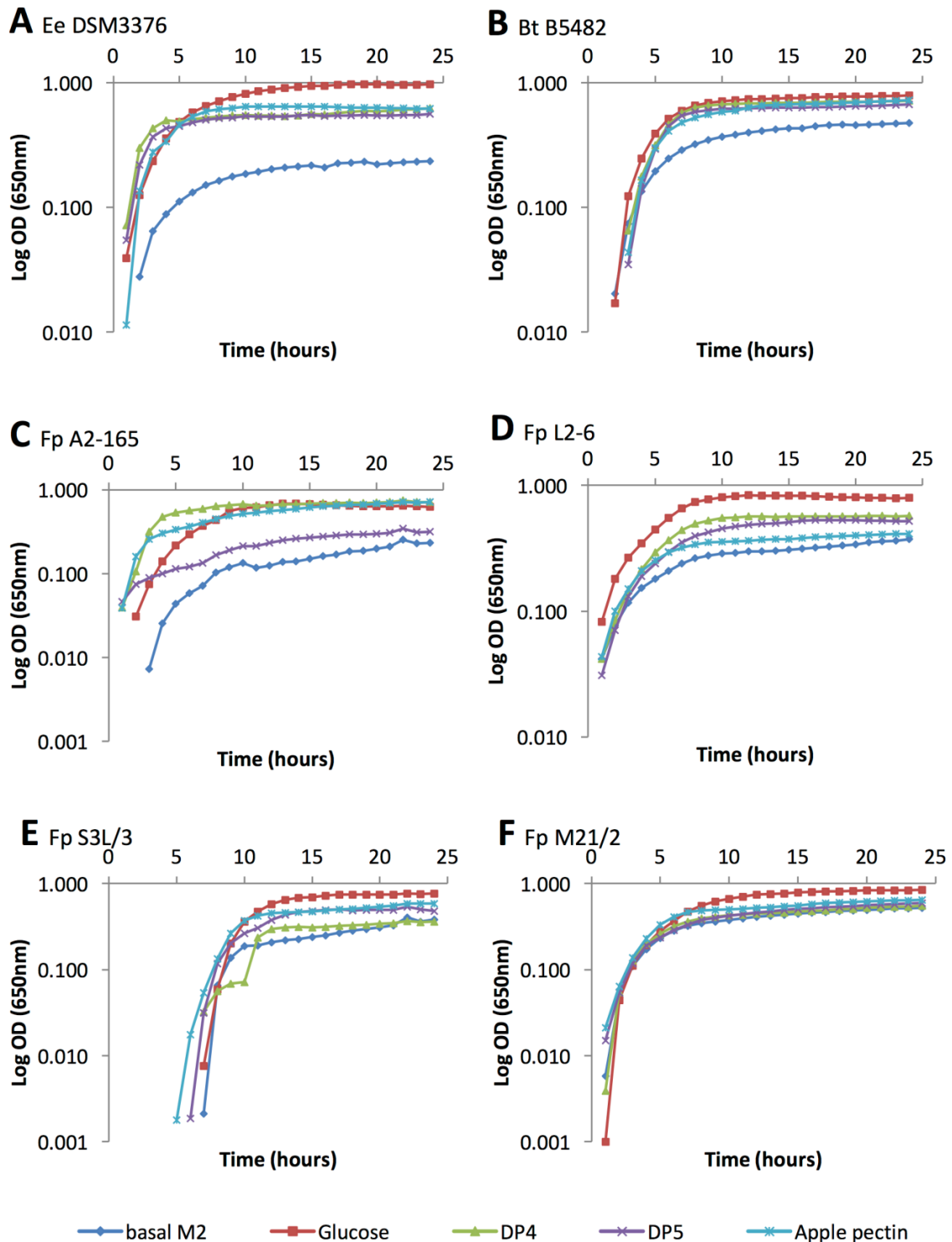
Figure 3. Growth stimulation by pectin oligosaccharides purified from sugar beet.
Comparison of growth in microtitre plates (mean ODs for triplicate wells) with different carbohydrate energy sources (0.2% final concentration); DP4 and DP5 refer to galacturonide chains of 4 and 5 residues, while basal M2 medium contains no added carbohydrate source. (A) *Eubacterium eligens* DSM3376, (B) *Bacteroides thetaiotaomicron* B5482, (C) *Faecalibacterium prausnitzii* A2-165, (D) *Faecalibacterium prausnitzii* L2-6, (E) *F.*

prausnitzii S3L/3, (F) *Faecalibacterium prausnitzii* M21/2, OD$_{650nm}$ values achieved in the absence of carbohydrates are assumed to reflect peptide utilization.
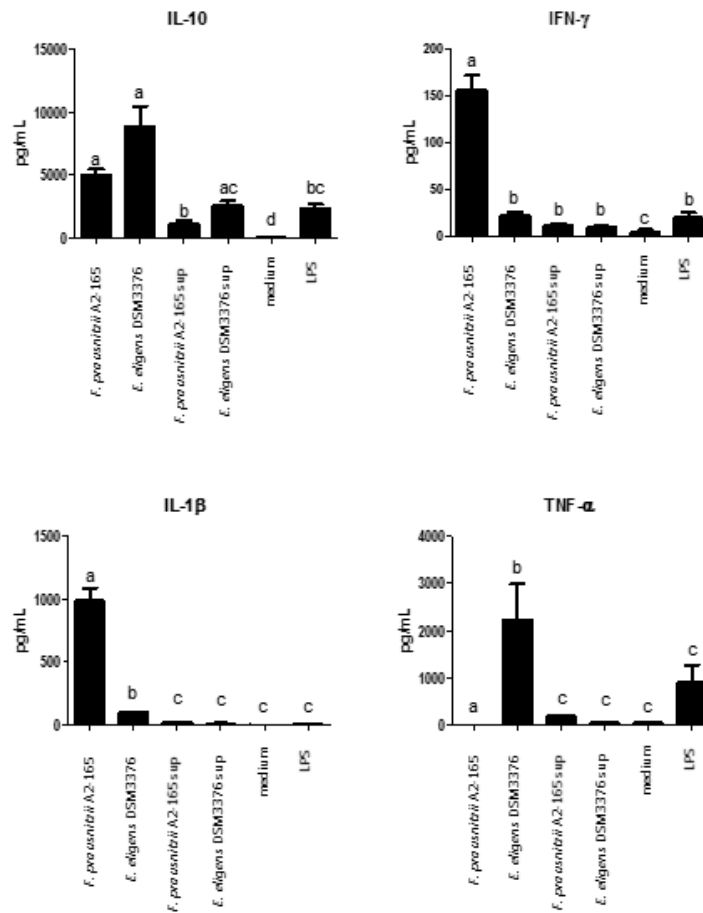
Figure 4. Cytokine profile of PBMCs stimulated with bacteria or *in vitro* culture supernatants of *E. eligens* DSM3376 or *F. prausnitzii* A2-165. Procedures are described in materials and methods. Mean amounts of cytokines (pg/ml) measured in PBMC culture supernatants are presented as the mean +/- standard error of the mean from three donors of duplicate wells. Different letters represent classes of statistically significant different responses

compared to each stimulation. The amount of IL-12 was below detection limit (5 pg/ml) for all samples.