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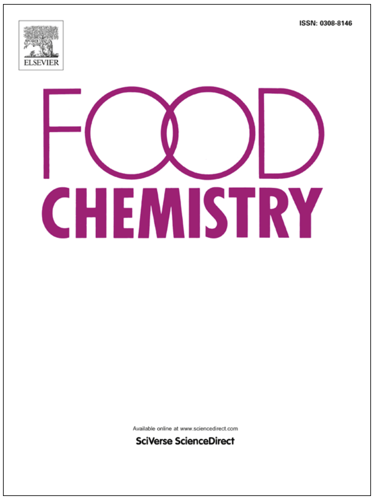

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Pectic oligosaccharide structure-function relationships: prebiotics, inhibitors of *Escherichia coli* O157:H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells

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Prebiotic and anti-adhesive pectic oligosaccharides

ABSTRACT

Shiga toxin (Stx)-producing, food-contaminating *Escherichia coli* (STEC) is a major health concern. Plant-derived pectin and pectic-oligosaccharides (POS) have been considered as prebiotics and for the protection of humans from Stx. Of five structurally different citrus pectic samples, POS1, POS2 and modified citrus pectin 1 (MCP1) were bifidogenic with similar fermentabilities in human faecal cultures and arabinose-rich POS2 had the greatest prebiotic potential. Pectic oligosaccharides also enhanced lactobacilli growth during mixed batch faecal fermentation. We demonstrated that all pectic substrates were anti-adhesive for *E. coli* O157:H7 binding to human HT29 cells. Lower molecular weight and deesterification enhanced the anti-adhesive activity. We showed that all pectic samples reduced Stx2 cytotoxicity in HT29 cells, as measured by the reduction of human rRNA depurination detected by our novel TaqMan-based RT-qPCR assay, with POS1 performing the best. POS1 competes with Stx2 binding to the Gb3 receptor based on ELISA results, underlining the POS anti-STEC properties.

Keywords:

STEC; orange pectic oligosaccharides; anti-adhesion; Shiga toxin 2; TaqMan RT-qPCR; rRNA depurination; HT29 cells

1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is a major health concern due to the debilitating hemolytic uremic syndrome which can occur when STEC-contaminated food is ingested. The Stx holotoxin consists of A and B subunits (Di, Kyu, Shete, Saidasan, Kahn, & Tumer, 2011). The doughnut-like structure formed by five of the Stx 7.7 kDa B-subunits binds to the neutral glycolipid globotriaosylceramide (Gb3) receptor terminated by α-Gal-(1-4)-ß-Gal on the human intestinal epithelial cell surface (Jacewicz, Clausen, Nudelman, Donohue-Rolfe, & Keusch, 1986). The interaction between Stx B pentamer and Gb3 results in the internalization of the Stx holotoxin by clathrin-mediated endocytosis (Sandvig, Grimmer, Lauvrak, Torgersen, Skretting, van Deurs, et al., 2002). The Stx A subunit is a glycosidase belonging to the ribosome-inactivating protein family that is capable of removing a specific adenine (depurination) in the conserved sarcin ricin loop (SRL) of the large 28S rRNA of mammalian cells, resulting in translation inhibition and cell death (Di, Kyu, Shete, Saidasan, Kahn, & Tumer, 2011). It is estimated by the Center for Disease Control that STEC O157:H7 causes more than 96,000 cases of diarrheal illness and 3,200 hospitalizations annually in the United States (Scallan, Hoekstra, ANgulo, Tauxe, Widdowson, Roy, et al., 2011). Presently there is no effective treatment for STEC-related food poisoning. There is considerable interest in developing dietary approaches to control food-contaminating pathogens.

Pectic oligosaccharides (POS) have potential as food ingredients that can control STEC pathogens. Plant-derived pectin and POS have attracted particular attention as they are abundant in biomass. Pectin consists of a galacturonic acid-rich backbone, known as homogalacturonan, that is partially methyl-esterified. Rhamnose residues interrupt the homogalacturonan to form rhamnogalacturonan I (RG I) and are the branch points for arabino-, galacto- and arabinogalacto-oligosaccharides. POS is obtained from pectin by enzymatic treatment and acid hydrolysis. POS from high methoxylated citrus pectin and from low methoxylated apple pectin protected human colonic HT29 cells from the toxic effects of *E. coli* O157:H7 Stx1 and Stx2 at 10 mg/ml (Olano-Martin, Williams, Gibson, & Rastall, 2003). However, the protective mechanism was not elucidated. Later, Rhoades, Manderson, Wells, Hotchkiss, Gibson, Formentin, et al. (2008) enumerated viable, attached STEC on HT29 cells and showed that POS provided 70% protection by inhibiting the adhesion of STEC at 2.5 mg/ml compared to non-POS-treated cells. These authors also found that the POS could reduce the cytotoxicity of Stx1 and Stx2 at concentrations of 0.01 to 1 μg/ml, respectively. However, the mechanism of activity was unclear since the POS did not contain α-Gal-(1-4)-ß-Gal.

POS are known for their prebiotic potential *in vitro*. The same POS that protected human colonic HT29 cells from the toxic effects of *E. coli* O157:H7 Stx1 and Stx2 was also bifidogenic (Olano-Martin, Gibson, & Rastell, 2002). POS from a variety of sources was bifidogenic if it contained arabino- and/or galacto-oligosaccharide side chains (Manderson, Pinart, Tuohy, Grace, Hotchkiss, Widmer, et al., 2005; Onumpai, Kolida, Bonnin, & Rastall, 2011). While Guggenbichler, De Bettignies-Dutz, Meissner, Schellmoser, & Jurenitsch (1997) originally reported that galacturonic acid disaccharides and trisaccharides had *E. coli* anti-adhesive activity, it remains unclear which pectic oligosaccharide structures are responsible, due to the diversity of pectic fractions reported to have this activity.

In previous studies, the cytotoxicity of Stx was measured as a function of neutral red uptake by the viable cells in the treated samples compared to the non-treated control sample. Molecular methods have been used to accurately measure the degree of rRNA damage from Stx depurination. The first such method is called dual primer extension, using two radioactively labelled oligo DNA primers to measure the levels of the broken rRNA and the total rRNA, respectively, in a single reverse transcription reaction (Di, Kyu, Shete, Saidasan, Kahn, & Tumer, 2011). Recently, a real-time RT-qPCR (reverse transcription-quantitative polymerase chain reaction) method with SYBR Green mix was developed to quantify the depurinated rRNA level in total RNA, based on the fact that reverse transcriptase usually incorporates an adenosine opposite to the abasic site on the template strand (Melchior & Tolleson, 2010). Thus, a T → A transversion is created when cDNA is synthesized by reverse transcriptase, using depurinated rRNA as the template. For this RT-qPCR depurination assay, two sets of primers are designed: one set close to the depurination site to measure total 28S rRNA and the other set to detect the altered sequence at the depurination site. Use of RT-qPCR to measure the level of depurinated rRNA among total RNA has greatly improved the accuracy of quantification. It has also saved time and obviated the use of radioactive materials. The RT-qPCR with SYBR Green method has been used to measure rRNA depurination caused by ricin and Stx (Melchior & Tolleson, 2010; Pierce, Kahn, Chiou, & Tumer, 2011).

In this study, we analyzed the carbohydrate structures of five different POSs from orange peel, compared their bifidogenic potentials and investigated their inhibitory effects on the adhesion of *E. coli* O157:H7 (ATCC43895) bacteria to HT29 cells. In addition, to study the inhibitory effect of POS on Stx cytotoxicity, we developed a novel

RT-qPCR method, using TaqMan probes to quantify the level of depurinated rRNA versus total rRNA as a measurement of Stx2 cytotoxicity in HT29 cells. TaqMan-based qPCR is practised for its higher specificity and sensitivity than SYBR Green-based qPCR. The TaqMan qPCR genotyping approach has been used to detect single nucleotide polymorphism (SNP) (Kamau, Alemayehu, Feghali, Tolbert, Ogutu, & Ockenhouse, 2012). As a T → A transversion is created when cDNA is synthesized using the depurinated RNA as a template, the cDNA population containing the T → A mutation can be considered as a cDNA with a single SNP. Our results show that the rRNA depurination resulting from Stx2 cytotoxicity can be sensitively measured by our TaqMan RT-qPCR method. We demonstrate the POS structures that are optimal for bifidogenic properties, inhibition of the adhesion of ATCC43895 to HT29 cells and reduction of the cytotoxicity of Stx2 in HT29 cells.

2. Materials and methods

2.1. POS

Orange peel POS (OpPOS) was prepared by pilot plant-scale acid hydrolysis of orange peel, according to Manderson, et al. (2005). The pectin was precipitated from the hydrolysate with isopropyl alcohol and removed by filtration. The filtrate containing OpPOS was desalted by 1,000 molecular weight cutoff nano-filtration. The OpPOS used here was a different batch produced at the same time as the material used by Manderson, et al. (2005). The differences in monosaccharide composition between the OpPOS batches were minor other than 8 × less glucose and 2 × more galacturonic acid in the OpPOS used here (Table 1) compared to that used previously (Manderson, et al., 2005). Pectic Oligosaccharide I (POS1), Pectic Oligosaccharide II (POS2), Modified Citrus Pectin I (MCP1) and Modified Citrus Pectin II (MCP2) were obtained from EcoNugenics, Inc. (Santa Rosa, CA, USA). The POS and MCP samples were produced by enzymatic treatment of citrus peel or commercial pectin.

2.2. Carbohydrate analysis

The POS and MCP monosaccharide composition was analyzed, following methanolysis (Manderson, et al., 2005), by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using a DX-500 (Dionex, Sunnyvale, CA, USA) system and a CarboPac PA-20 column operated at 0.5 ml/min, as described previously (Hotchkiss, Nunez, Strahan, Chau, White, Marais, et al., 2015). The HPAEC-PAD mobile phase consisted of 14 mM NaOH for 13 min, followed by a 0–120 mM CH3COONa gradient in 100 mM NaOH for 17 min and it was returned to 14 mM NaOH for 40 min prior to the next injection. Molecular weight (MW) was determined by high pressure size exclusion chromatography (HPSEC), with three TSKgel GMPWXL (Tosoh Bioscience, Tokyo, Japan) columns and four detectors (HELEOS II multi-angle laser light scattering, refractive index, 255-V2 differential pressure viscometer; Wyatt Technology, Santa Barbara, CA, USA) and a UV-1260 Infinity spectrophotometer (Agilient Technologies, Santa Clara, CA, USA), as reported previously (Qi, Chau, Fishman, Wickham, & Hotchkiss, 2014). MW values reported are weight average molar mass values. The degree of methyl esterification was determined as described previously (Fishman, Chau, Cooke, & Hotchkiss, 2008).

2.3. In vitro batch fermentation

Basal medium ingredients (per litre) were: 2.0 g peptone water, 2.0 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$, 2.0 g $NaHCO_3$, 2 ml Tween 80, 0.05 g haemin, 10 µl vitamin K1, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4 ml resazurin (0.05 g/l). Medium was sterilized at 120 ^{o}C for 20 min before aseptically dispensing into the sterile fermenters. Substrates were used at 1% (w/v) as the sole carbon source. Inulin ST (Beneo-Orafti, Tienen, Belgium) was used as a positive control. Faecal samples from five healthy adults (3 male, 2 female, mean age of 30.0±7.2 years old) who had not consumed prebiotic or probiotic products, nor had received antibiotic treatment within 3 months before study were obtained *in situ* in the Department of Food and Nutritional Sciences, The University of Reading. Samples were kept in an anaerobic cabinet and processed within 10 min. Faecal slurries (10% w/w) in 0.17 M phosphate-buffered saline (PBS), pH 7.3 (Oxoid, Basingstoke, UK) were prepared and were homogenized in a stomacher (Stomacher 400, Seward, UK) at normal speed for 2 min. The inoculum size was 10% v/v. pH was regulated at 6.80±0.10 with a pH controller (Fermac 260, Electrolab, Tewkesbury, UK). Fermentation samples were taken at 0, 10, 24, 36 and 48 h. Samples were analyzed for bacterial populations and concentration of short chain fatty acids (SCFA). All experiments were performed in compliance with the laws and guidelines at the University of Reading, UK.

2.4. SCFA analysis by HPLC

The samples from batch cultures were centrifuged at 13,000 *g* for 10 min to obtain the supernatant. The clear solution was kept at -20 ^{o}C prior to further analysis. Before analysis by HPLC, the samples were centrifuged at 13,000 *g* for 10 min. The supernatant was filtered through 0.2 μm pore size syringe filters (Millipore, UK). The column was an ion-exclusion REZEX-ROA organic acid column (300 × 7.80 mm; Phenomenex, Cheshire, UK) maintained at 84 °C. The eluent was 0.0025 mM H_2SO_4, flow rate of 0.6 ml/min. Concentrations of the separated organic acids were calculated from calibration curves of acetic, propionic, butyric, formic and lactic acids at concentrations of 6.25 to 120 mM, and results were expressed in mmol/ml.

2.5. Bacterial enumeration by fluorescence in situ hybridization (FISH)

Enumeration of the target faecal bacteria groups was achieved by FISH with fluorescently labelled 16S rRNA probes according to the method described by Vulevic, Drakoularakou, Yaqoob, Tzortzis, & Gibson (2008). The 16S rRNA-targetted oligonucleotide probes used were Lab158 (Harmsen, Elfferich, & Schut, 1999), Bif164 (Langendijk, Schut, Jansen, Raangs, Kamphuis, Wilkinson, et al., 1995), Bac303 (Manz, Amann, Ludwig, Vancanneyt, & Schleifer, 1996), Erec482 (Franks, Harmsen, Raangs, Jansen, Schut, & Welling, 1998), Chis150 (Franks, Harmsen, Raangs, Jansen, Schut, & Welling, 1998) and Ato291 (Harmsen, Wildeboer-Veloo, Grijpstra, Knol, Degener, & Welling, 2000) for the group of *Lactobacillus*/*Enterococcus*, *Bifidobacterium*, *Bacteroides*/*Prevotella*, *Clostridium coccoides–Eubacterium rectale*, *Clostridium histolyticum* and *Atopobium* cluster, respectively. The probe-hybridized bacterial cells were counted at 565 nm, using fluorescence microscopy. A total bacterial count was obtained by staining with 4'6-diamidino-2-phenylindole (DAPI). Bacterial cells were counted at 461 nm, using UV light for excitation. A minimum of 15 fields of view were counted for each sample. The number of cells obtained is expressed as log10 cells/ml.

Statistical analysis was performed using SPSS for Windows, version 17.0. One-way analysis of variance (ANOVA) and Tukey's *posthoc* test were used to determine significant differences among the bacterial group populations and SCFA concentrations among the different substrates. A paired independent t-test was also used to determine significant changes for each bacterial group concentration at inoculation and subsequent sampling point. Differences were considered to be significant when $p<0.05$.

2.6. Microbiological media and chemicals

Pre-formulated, dehydrated tryptic soy agar medium and the following chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and prepared according to the manufacturer's instructions: PBS tablets (pH ± 0.2, 0.1 mol/l), non-essential amino acid solution, trypsin-EDTA solution and Dulbecco's modified Eagle medium with GlutaMAX-1 (DMEM). PBS was prepared according to the manufacturer's instructions and filter-sterilized with a 0.2 µm syringe filter. Faetal bovine serum (FBS) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.7. Bacterial cultures

Working cultures of *E. coli* O157:H7 strain ATCC43895 (ATCC, Manassas, VA, USA) were prepared by inoculating the bacteria on a count agar plate and incubating the plate for 18-24 h at 37 ºC. *E. coli* broth cultures for adhesion assays were grown in DMEM supplemented with 5% (v/v) FBS and 1% (v/v) nonessential amino acid solution (SDMEM) and incubated at 37 ^{o}C for 18-24 h. The overnight culture was then inoculated 1% (v/v) into fresh SDMEM and incubated for a further 18-24 h under the same conditions. On the day of the assay, a 10% (v/v) inoculum was again inoculated into pre-warmed SDMEM and incubated for 4 h at 37 ºC.

2.8. Cell cultures

HT29 human colon adenocarcinoma epithelial cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 5% FBS and 1% SDMEM, plus 20 units/ml of penicillin, and 20 µg/ml of streptomycin at 37 ^{o}C with 5% CO_2. Cells were grown in 25-cm^2 tissue culture flasks until reaching confluence, split according to the European Collection of Cell Cultures-recommended method and stored in aliquots in liquid nitrogen. These aliquots were used to seed 25 cm^2 flasks which, after growth, were split into 12-well tissue culture plates. Cells were grown to confluence before being used for the adhesion assays.

2.9. Bacterial adhesion assay

Adhesion assays with the *E. coli* strains were carried out as follows: a culture of the test strain was prepared as described above, and then diluted 1:500 in PBS. The viable count of the diluted suspension was determined by spread-plating onto plate count agar, with decimal dilution being carried out in PBS buffer as appropriate. POSs were dissolved in PBS (5 mg/ml) and sterilized by passing through a 0.2 µm syringe filter. The carbohydrate solutions were further diluted in sterile PBS as required. The SDMEM was aspirated into a 12-well tissue culture plate with near confluent monolayers of HT29 cells, prepared as described above. The monolayers were washed by pipetting in 1 ml of sterile PBS per well, swirling by hand, and then aspirating. A 0.5 ml aliquot of POS solution was added to the well, followed by 0.5 ml of bacterial suspension in PBS. Un-supplemented PBS was substituted for POS solution in the control well. All assays were performed in triplicates. The plates were swirled by hand to mix and incubated at 37 ºC for 2 h.

After incubation, the bacterial suspension was aspirated from the wells. A 1 ml aliquot of PBS was added to each well, the plate was swirled briefly by hand, and the PBS was removed. The washing step was repeated two more times. A 70 µl aliquot of trypsin-EDTA solution was added to each well, the plate was rocked to ensure even coverage, and then it was incubated at 37 ºC for 5 min. A 1 ml aliquot of PBS was then pipetted into each well and pipette-mixed until the monolayer was completely dislodged and clumps dissolved, as determined visually. Bacteria in cell suspension were then enumerated by plate-counting on count agar plates with decimal dilutions performed in PBS as required. All plates were incubated at 37 ºC for 18-24 h before colonies were enumerated. Viable counts were calculated for all wells and are expressed as CFU (colony forming unit)/ml. The anti-adhesion activity of POS was assessed as the percentage of viable counts in the POS-treated samples compared to the untreated samples. For each test, the mean and the standard error of the triplicate wells were calculated. Statistical significances were determined by one-way analysis of variance, using ANOVA software.

2.10. Cytotoxicity of Stx2 in HT29 cells

Stx2 holotoxin was acquired from BEI Resources (Manassas, VA). HT29 cells grown to 90% confluence were treated with 5 ng of Stx2 in 100 μl SDMEM per well (final Stx2 concentration was 50 ng/ml) in the 96-well plate for 24 h (Pang, Park, Wang, Vummenthala, Mishra, McLaughlin, et al., 2011). To isolate total RNA from treated and untreated HT29 cells, the RLT lysis buffer from the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was added directly to each well after the medium was aspirated. Total cell RNA was extracted following the manufacturer's protocol and quantitated by a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA).

2.11. Dual primer extension assay to measure rRNA depurination due to Stx2 cytotoxicity in HT29 cells

The dual primer extension method has been used extensively to determine the levels of rRNA depurination caused by Stx (Di, Kyu, Shete, Saidasan, Kahn, & Tumer, 2011) and ricin from *Ricinus communis* (castor bean) (Li, Baricevic, Saidasan, & Tumer, 2007). In brief, a primer (P1, 28S) was designed to anneal to the 5' end of human 28S rRNA (GenBank accession # NR_003287) and would produce a 99-base single-stranded (ss) cDNA in a RT (reverse transcription) reaction. Another primer (P2, Dep) was designed to anneal just upstream of the depurination site in the sarcin ricin loop (SRL) of 28S rRNA, resulting in a 72-base ss cDNA RT product if 28S rRNA was depurinated (Fig. 1). Both the 28S and Dep primers were end-labelled with ^{32}P-ATP by T4 kinase and used together in an RT reaction by Superscript II (Life Technologies/Thermo Fisher, Waltham, MA, USA), using total RNA as the template. The RT products were separated on a 7M urea-containing 5% polyacrylamide gel by electrophoresis. The autoradiogram was scanned and recorded by a phosphoimager (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.12. Development of the TaqMan RT-qPCR method to measure rRNA depurination as a result of Stx2 cytotoxicity in HT29 cells

Based on the nucleotide sequence of human 28S rRNA (accession # NR_003287), and using the PrimerExpress software of Applied Biosystems (Life Technologies/Thermo

Fisher, Waltham, MA, USA), one set of primers (HSRL F and HSRL R) was designed to amplify the 71-bp spanning the depurination site at the 3' end. Two TaqMan probes (HSRL and HSRLm) were designed at the adenine depurination site to quantitate the non-depurinated and depurinated 28S rRNA levels in HT29 cells, taking advantage of the T to A transversion (mutation). Another set of primers and a TaqMan probe (H28S) were designed to amplify the 62-bp at the 5' end of the 28S rRNA to measure the total 28S rRNA levels. The oligonucleotide primers were synthesized by Sigma Aldrich (St. Louis, MO, USA). The TaqMan probes were synthesized by Applied Biosystems (Life Technologies/Thermo Fisher, Waltham, MA, USA). The positions of these primers and TaqMan probes are illustrated in Fig. 1 and the sequences are shown in Supplement 2.

Reverse transcription reaction was carried out with 10 ng of total RNA, using the High Capacity cDNA Kit (Life Technologies/Thermo Fisher, Waltham, MA, USA) and random primers. The qPCR analysis was performed with the 2× TaqMan Master Mix (Life Technologies/Thermo Fisher, Waltham, MA, USA) and the designed primer and TaqMan probe sets with final concentrations of 900 nM for each primer and 250 nM for each probe in, totally, 10 μl. The reaction cycles were as follows: 95 ^{o}C, 20 min, 1 cycle; 95 ^{o}C, 1 min, 60 ^{o}C, 2 min, 40 cycles.

2.13. Treatment of HT29 cells with Stx2 and POS

HT29 cells were seeded into a 24-well plate (Thermo Fisher, Waltham, MA, USA) and grown to 90% confluence. Before treatment with POS and Stx2, cells were washed once with PBS buffer after aspirating the culturing medium. POS with different concentrations was incubated with 5 ng of Stx2 in 100 μl volume of DMEM for 1 h at room temperature. These "pre-culture (PC)" POS and Stx2 mixtures were added to HT29 cells which were then incubated at 37 °C with 5% CO_2 for 24 h. Alternatively, POS at different concentrations was mixed with 5 ng of Stx2 in 100 μl volume of DMEM and added directly to HT29 cells without pre-incubation, designated as "co-culture (CC)" samples. Total RNA was isolated from treated HT29 cells using the RNeasy Mini Prep Kit (Qiagen, Hilden, Germany) and quantitated by a Nanodrop Spectrophotometer (Thermo Fisher, Waltham, MA, USA).

2.14. Competition ELISA

To understand the protective mechanism of POS on HT29 cells against Stx2, competition ELISA (enzyme-linked immunosorbent assay) was conducted to evaluate if POS1 could compete with Stx2 to bind to the Gb3 (globotriaosylceramide) receptor on the cells. Gb3 was purchased from Matreya LLC. (State College, PA, USA), diluted in 100% methanol, and added to each well of the 96-well plate (50 ng/well). The plate was incubated at room temperature for 2 h until the methanol was evaporated. The wells were blocked with 300 μl of 5% dry milk prepared in PBS and incubated at 37 °C for 1 h. For pre-treatment of Stx2, POS1 at 0.02, 0.2 and 1 mg/ml was mixed with 5 ng of Stx2 and incubated at 37 °C for 1 h. Otherwise, POS1 at these concentrations was mixed with 5 ng of Stx2 and added directly to each well in 100 μl of PBS, followed by incubation at 37 °C for 1 h. Polyclonal antibody, specific to the Stx B-subunit, was obtained from BEI Resource, diluted to 1.3 μg/ml with 5% dry milk in PBS, and added to the wells (80 μl/well), followed by incubation at 37 °C for 1 h. Mouse monoclonal antibody against rabbit IgG, coupled with alkaline phosphatase (Sigma Aldrich, St. Louis, MO, USA), was diluted by 1:1000 in 5% dry milk/PBS and added to the wells (50 μl/well). The plate was incubated at 37 °C for 1 h. Lastly, the SIGMAFAST p-nitrophenyl phosphate tablet (Sigma Aldrich, St. Louis, MO, USA) was dissolved in SIGMAFAST Tris Buffer (Sigma Aldrich, St. Louis, MO, USA) and added to the wells (50 μl/well). The plate was incubated at room temperature until the yellow colour became apparent and read at 405 nm with the Synergy 4 plate reader (BioTek, Winooski, VT, USA).

3. Results and discussion

3.1. POSs

Galacturonic acid was the major saccharide residue in four of the pectic fractions (POS1, POS2, MCP1 and MCP2), ranging from 49.2% (w/w) to 79.0% (Table 1). The galacturonic acid content of OpPOS was 22.8%, which meant it was enriched in RG I compared to the other pectic fractions that had 2-3.5× more homogalacturonan, based on galacturonic acid:rhamnose ratios (Table 1). Significant arabino-oligosaccharide branches were present in the OpPOS and POS2 RG I while the galacto-oligosaccharide branches were similar for all samples, based on monosaccharide ratios (Table 1). POS2 reproduced the OpPOS fraction structure published previously (Manderson, et al., 2005) in which the rhamnogalacturonan is heavily substituted with arabinan and arabinogalactan. Arabino- and galacto-oligosaccharide structures are important for the prebiotic properties of POS (Onumpai, Kolida, Bonnin, & Rastall, 2011). MCP contains unsaturated oligogalacturonic acids as well as rhamnogalacturonan II produced by enzymatic hydrolysis of commercial pectin to provide anti-cancer, immuno-stimulatory and heavy metal-binding properties (Eliaz, Hotchkiss, Fishman, & Rode, 2006; Maxwell, Colquhoun, Chau, Hotchkiss, Waldron, Morris, et al., 2015; Ramachandran, Wilk, Hotchkiss, Chau, Eliaz, & Melnick, 2011). Unsaturated oligogalacturonic acids with a DP of 2-7 were the intermediate degradation products of citrus pectin during human faecal bacterial fermentation (Dongowski & Lorenz, 1998). The degree of methyl esterification was low in MCP1 and MCP2 (5.3% and 3.3%), intermediate in POS1 and POS2 (40.1% and 42%), and high in OpPOS (66.3%) (Table 1). MCP1 had the lowest MW (9.2×10^3) and MCP2 had the second lowest MW (17.1×10^3), followed by POS1, OpPOS and POS2 (811×10^3) (Table 1). However, the OpPOS and POS2 weight average molar masses were likely higher than their molecular weights reported in Table 1, due to a high light-scattering signal that eluted earlier than the refractive index signal in the HPSEC chromatograms (data not shown). This typically indicates aggregation of smaller pectic components that may not be covalently linked together as has been reported previously for pectin (Fishman, Chau, Cooke, & Hotchkiss, 2008).

3.2. SCFA production

The average SCFA produced from mixed batch fermentation, using faeces from five donors, is shown in Table 2. Total organic acids increased sharply by 10 h, reaching a maximum after 36-48 h for POS1, POS2, MCP1 and inulin. Acetate, propionate and butyrate concentrations increased with fermentation time until 48 h. Lactate and formate are fermentation intermediates and they completely disappeared after 24 h. Acetate was previously reported as the main SCFA from pectin oligosaccharide fermentation, followed by propionate and butyrate (Dongowski & Lorenz, 1998; Titgemeyer, Bourquin, Fahey, & Garleb, 1991). Butyrate levels were significantly higher with inulin compared to the other substrates at 24 h, and inulin produced more butyrate than POS1 and MCP1 at 36 h. High standard deviation values made it impossible to distinguish between butyrate levels produced by all substrates at 48 h and other times during fermentation. Butyrate is considered to be important for colonic health and function (Hamer, Jonkers, Venema, Vanhoutvin, Troost, & Brummer, 2008). All pectic substrates evaluated consistently produced butyrate and it is anticipated that they would promote colonic health. Overall, POS1, POS 2 and MCP1 showed similar fermentabilities to inulin, based on the average total organic acids, acetate, propionate, lactate and formate concentrations. OpPOS had a fermentability similar to fructo-oligosaccharides, producing acetate, butyrate, lactate and propionate in that concentration order for 24 h of human faecal fermentation (Manderson, et al., 2005). The degree of methyl esterification, sugar composition and molecular weight did not influence the POS or MCP fermentability, as reflected by the similar SCFA yield and profile.

3.3. Microbiota changes

The microbial profiles in the batch cultures are presented in Table 3. Inulin, POS1, POS2 and MCP1 significantly increased Bif164 numbers, with numbers remaining elevated at 48 h (36 h for POS1) compared to time 0. Inulin and POS2 were significantly more bifidogenic than were POS1 and MCP1 throughout the fermentation period. After 24 h of fermentation, inulin was more bifidogenic than was POS2. The bifidogenic properties of OpPOS were similar to fructo-oligosaccharides, as reported previously (Manderson, et al., 2005). A rise in Lab158 level was obtained with POS2 through 48 h, inulin through 36 h and POS1 through 24 h of fermentation.

Our results confirm the previously reported correlation between arabinose content and bifidogenic properties (Manderson, et al., 2005; Onumpai, Kolida, Bonnin, & Rastall, 2011) since POS2 and OpPOS exerted higher stimulation of Bif164 than did POS1 and MCP. The pectic oligosaccharide MW and DE did not affect their bifidogenic properties since POS1 and MCP1 were equally bifidogenic. However, all pectic substrates evaluated were bifidogenic. Therefore, structural diversity in pectic prebiotics is possible as long as significant arabino- and galacto-oligosaccharide content is present. Bifidogenic POS activity was the only prebiotic property previously reported (Manderson, et al., 2005; Onumpai, Kolida, Bonnin, & Rastall, 2011) and this is the first report of POS selecting for higher lactobacillus levels during mixed batch faecal fermentation.

Erec482 levels increased with MCP1 through 36 h and with inulin at 24 h (Table 3). OpPOS was previously reported to enhance Erec482 counts and butyrate production during human faecal fermentation and *Eubacterium rectale* is known to produce butyrate (Manderson, et al., 2005). In our analysis, MCP1 Erec482 numbers did not correlate with butyrate concentration, since MCP1 produced less butyrate than inulin did. In other treatments, Erec482 counts remained at the inocula levels. MCP1 might increase non-butyrate producing-bacteria detected by the Erec482 probe and increases in butyrate levels produced by other substrates may be due to faecal bacteria besides *Eubacterium rectale*.

The significant increase in Bac303 numbers on all substrates through 48 h of fermentation (Table 3) agreed well with data published previously (Onumpai, Kolida, Bonnin, & Rastall, 2011). Bacteroides have the ability to utilize pectin and many bacteroides strains isolated from human faeces can produce various pectinolytic enzymes, including polygalacturonase, pectin methylesterase, extracellular and cell-associated pectate lyase (Bayliss & Houston, 1984; Dekker & Palmer, 1981; Jensen & Canale-Parola, 1986). Chis150 numbers rose on MCP1 through 36 h, POS2 through 24 h and inulin at 10 h. Bac303 and Chis150 groups include pathogens. In a previous *in vitro* study of POS (Manderson, et al., 2005; Olano-Martin, Williams, Gibson, & Rastall, 2003), Chis150 and Bac303 numbers remained at the initial level while Bif164 counts significantly increased. The different results might be explained partly by carbohydrate structural differences or microbial variation in faecal samples.

Ato291 levels increased with MCP1 and inulin through 48 h, POS2 through 36 h and POS1 at 48 h. *Atopobium* is grouped within the actinomycetes, which can produce lactic acid (Jovita, Collins, Sjoden, & Falsen, 1999); it is one of the predominant bacterial groups in adult faeces (Harmsen, Wildeboer-Veloo, Grijpstra, Knol, Degener, & Welling, 2000), and has been observed in a human trial of very long chain inulin from globe artichoke (DP 50-103) (Costabile, Kolida, Klinder, Gietl, Bauerlein, Frohberg, et al., 2010). However, the role of this bacterium in human gut health is not yet established.

The total bacterial concentrations of the samples obtained from the pH-temperature controlled stirred-batch fermentation, using POS1, POS2, MCP1 and inulin as carbon sources, were measured by DAPI staining. The data supported our findings shown in Table 3 and are presented in Supplement 1.

3.4. Anti-bacterial adhesion activity of POS in HT29 cells

We previously showed that OpPOS inhibited the adhesion of enteropathogenic *E. coli* and verotoxigenic *E. coli* strains to HT29 cells, by 50%, at a concentration of 0.15 to 0.46 mg/ml (Rhoades, et al., 2008). In this study, we showed that five pectic oligosaccharides (POS1, POS2, OpPOS, MCP1 and MCP2) displayed anti-adhesion activity to some degree against the Shiga toxin-producing *E. coli* O157:H7 in HT29 cells. Our results confirmed the previously reported correlation between oligogalacturonic acid content and inhibition of *E. coli* adhesion (Guggenbichler, De Bettignies-Dutz, Meissner, Schellmoser, & Jurenitsch, 1997) since POS1 had the highest anti-adhesion activity throughout the 0.005 – 5 mg/ml concentration range (Table 4) and it had a high GalA:Rha ratio (Table 1). MCP1 and MCP2 also had high GalA:Rha ratios and exhibited anti-adhesion activity equivalent to POS1 in the 0.8-2.5 mg/ml concentration range. These pectic substrates had similar monosaccharide compositions, lowest molecular weights and low degree of esterification with the exception of POS1, which had intermediate degree of esterification, indicating that smaller, deesterified structures are important for anti-adhesion activity. OpPOS had the lowest GalA:Rha ratio of the pectic substrates but minor amounts of unsaturated oligogalacturonic acids present in this sample may have contributed to its anti-adhesion activity in the lower oligosaccharide concentration range (0.005 - 0.5 mg/ml) where the greatest anti-adhesion activity (50 - 90%

inhibition of E. coli O157:H7 adhesion) was observed. The relatively high degree of esterification in OpPOS limited its anti-adhesive activity at 0.5 mg/ml and higher concentrations.

The adhesion of *E. coli* STEC strains to human cells involves multiple mechanisms, including intimin (McKee, Melton-Celsa, Moxley, Francis, & O'Brien, 1995), *E. coli* common pilus (Rendon, Saldana, Erdem, Monteiro-Neto, Vazquez, Kaper, et al., 2007) and type IV pilus (Xicohtencatl-Cortes, Monteiro-Neto, Ledesma, Jordan, Francetic, Kaper, et al., 2007). High *E. coli* O157:H7 anti-adhesion activity, correlated with the lower range of oligosaccharide concentrations, has been reported previously for OpPOS (Rhoades, et al., 2008) and cranberry xyloglucan (Hotchkiss, et al., 2015). We recently reported that cranberry xyloglucan oligosaccharides were inhibitory to the adhesion of an STEC strain ATCCBAA-1883 on HT29 cells at low concentrations (0.001-0.1 mg/ml) (Hotchkiss, et al., 2015). However, xyloglucan oligosaccharides had much higher affinity for type 1 fimbriated uroepithelial *E. coli* that are specifically inhibited by mannose-containing oligosaccharides (Hotchkiss, et al., 2015). Pectin-like acidic polysaccharide from the root of *Panax ginseng,* which consists primarily of galacturonic and glucuronic acids along with rhamnose, arabinose, and galactose as minor components, exerted a selective anti-adhesive effect against pathogenic bacteria *Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes* and *Staphylococcus aureus* while having no effects on beneficial and commensal bacteria *Lactobacillus acidophilus*, *Escherichia coli* or *Staphylococcus epidermidis* (Lee, Shim, Lee, Kim, Chung, & Kim, 2006). Our results with POS1, POS2, MCP1, and MCP2 confirmed other reports (Olano-Martin, Williams, Gibson, & Rastall, 2003; Rhoades, et al., 2008) that pectic oligosaccharides block the specific interaction required for adhesion of P-fimbriated *E. coli* to human epithelial cells. It is known that P-fimbriated *E. coli* and Stxs, produced by STEC, utilize the same α-Gal-(1-4)-β-Gal terminal oligosaccharide receptor to adhere to epithelial cells. The mechanism of POS inhibition of P-fimbriated *E. coli* adhesion remains unknown, since α-Gal-(1-4)-β-Gal was not observed in the structures of our POSs, and receptor mimicry is unlikely to be involved.

3.5. rRNA depurination by Stx2 was measured by the novel TaqMan RT-qPCR method

To investigate whether the anti-*E. coli* O157:H7 adhesion activity of orange POSs in HT29 cells could result in reduction of Stx cytotoxicity, we first developed a TaqMan probe-based RT-qPCR analysis to measure the rRNA depurination caused by pure Stx2 holotoxin. Due to the specific removal of a single adenine (depurination) from the SRL of the large subunit of rRNA by Stx, we took advantage of the creation of the T → A transversion when cDNA is synthesized by reverse transcriptase, using depurinated rRNA as the template, and designed two TaqMan probes. The first was the SRL probe (T1) that could measure the level of un-depurinated, intact rRNA when paired with depurination forward (Dep F, P5) and depurination reverse (Dep R, P6) primers (Fig. 1a and Supplement 2). The second TaqMan probe was the SRLm probe (T3) that could measure the depurinated rRNA level when paired with Dep F and Dep R primers (Fig. 1a and Supplement 2).

After HT29 cells were treated with pure Stx2 holotoxin (50 ng/ml) for 24 h, total RNA was isolated and cDNA was produced by reverse transcriptase, using random primers. Stx2 at 50 ng/ml has been shown to cause rRNA depurination of Vero cells (Pang, et al., 2011); we used the established dual primer extension method to determine whether Stx2-treated HT29 cells were depurinated under the experimental conditions. As shown in Fig. 1b, the Stx2-treated HT29 cells produced the predicted depurination band of 72 bases by the P2 (Dep) primer as well as the control band of 99 bases by the P1 (28S) primer, compared to the untreated cells that produced only the control 28S band.

To measure rRNA depurination caused by Stx2 in HT29 cells by our novel TaqMan probe-based RT-qPCR method, the amplification efficiencies of Dep F (P5)/Dep R (P6) primers and SRL (T2), SRLm (T3) TaqMan probes for non-depurinated and depurinated rRNA, and 28S F (P3)/28S R (P4)/28S probe (T1) for total 28S rRNA, were first validated. The cDNAs from the untreated and Stx2-treated cells were serial-diluted. The cDNA serial dilutions from the untreated cells were amplified by Dep F/Dep R and the SRL probe, and the cDNA serial dilutions from the Stx2-treated cells were amplified by Dep F/Dep R and the SRLm probe. Additionally, the cDNA serial dilutions from the untreated cells were amplified by the 28S F/28S R/28S probe. Fig. 1c shows that the intercept and R^2 value were -3.476 and 0.9995 for Dep F/Dep R/SRL, -3.46 and 0.9981 for Dep F/Dep R/SRLm, and -3.652 and 0.9988 for 28S rRNA (as the endogenous control), indicating that the non-depurinated rRNA, depurinated rRNA and total 28S rRNA were equally efficiently amplified by our designed primers and TaqMan probes.

The 1:100-diluted cDNA samples from the Stx2-treated and untreated (control, Ctr) HT29 cells were amplified by the primers/TaqMan probe sets for the three gene targets, SRLm (representing the depurinated rRNA), SRL (representing the non-depurinated rRNA) and 28S (representing the total rRNA). The average threshold cycle

(Ct) numbers from triplicates were used to calculate the fold-change of SRLm and SRL levels in Stx2-treated HT29 cells (Stx2 sample) compared to the untreated (Ctr sample), relative to the level of 28S (Supplement 3). Because not all of the rRNA molecules are depurinated by Stx2, the ratio of the SRLm level over that of SRL would represent the level of rRNA depurination. A higher ratio of SRLm/SRL indicates a higher level of rRNA depurination in the cells. Supplement 3 shows that the ratio of SRLm/SRL in the Stx2-treated cells was 39.7.

Following ingestion of STEC, the bacterial cells colonize human intestines and produce Stxs, which then interact with glycolipid Gb3 receptor and become internalized into the intestinal epithelial cells (Hurley, Jacewicz, Thorpe, Lincicome, King, Keusch, et al., 1999). Therefore, prebiotics, food constituents or inhibitors that can prevent the colonization and the Stx internalization will be of great advantage to prevent the detrimental effects of STEC and Stxs. Our results showed that the primer/TaqMan probe sets that we designed for the SRLm, SRL and 28S gene targets were able to sensitively quantify the levels of rRNA depurination caused by Stx2 in HT29 cells (Fig. 1a and Supplement 3).

3.6. Orange POSs reduced the Stx2 rRNA depurination in HT29 cells

HT29 cells have been shown to be intoxicated by pure Stx holotoxins (Olano-Martin, Williams, Gibson, & Rastall, 2003; Rhoades, et al., 2008). To determine if orange POSs could directly interact with Stx2 and inhibit its internalization into cells, we incubated orange POSs with Stx2 and then measured the reduction of rRNA depurination in HT29 cells. For this testing, we selected POS1 and MCP2 for the best performance in the anti-bacterial adhesion assay; we also selected OpPOS as the acid preparation of orange POS, and we selected POS2 as the one with the lowest anti-bacterial adhesion activity (Table 4). For the “pre-culture (PC)”samples, POS1, MCP2, OpPOS and POS2, at different concentrations, were incubated with 5 ng of Stx2 for 1 h at room temperature before being added to HT29 cells. For the “co-culture (CC)” samples, these four orange POSs were mixed with 5 ng of Stx2 and added directly to HT29 cells. The addition of POSs alone to HT29 cells did not cause cell death, as measured by the MTS assay (Promega, Madison, WI) (data not shown).

After 24 h of treatment, HT29 cells were lysed; total RNAs were isolated, RT- and qPCR reactions were performed with the primer/TaqMan sets for SRLm, SRL and 28S gene targets (Supplement 2). The ratios of SRLm/SRL were calculated, as mentioned above, and compared to that of the Stx2-treated cells. Our results showed that all four POSs reduced the Stx2 rRNA depurination when co-cultured with Stx2 in HT29 cells (Table 5). The best performer in anti-adhesion assay, POS1, displayed the highest reduction in the Stx2 rRNA depurination in a dose-dependent manner. More than 44% reduction of rRNA depurination was achieved when POS1 at 100 μg/ml concentration was co-cultured with Stx2. POS1 was also the only one that could reduce Stx2 rRNA depurination when pre-incubated with Stx2 at concentration of 10 and 100 μg/ml; however, at much lower levels, 3.43% and 6.59%, respectively.

3.7. Orange POS1 competed with Stx2 to bind to Gb3 on HT29 cells

The pentamer of StxB subunits binds to the glycolipid globotriaosylceramide (Gb3) receptor on the human cell surface to get StxA subunit internalized (Di, Kyu, Shete, Saidasan, Kahn, & Tumer, 2011; Jacewicz, Clausen, Nudelman, Donohue-Rolfe, & Keusch, 1986). Our results, above, demonstrated that all four POSs reduced the Stx2 depurination of HT29 rRNA when co-cultured with Stx2, suggesting that POSs might compete with Stx2 for binding sites on the HT29 cell surface, thus blocking the entry of Stx2A into cells. We devised a competition ELISA assay to test whether POS1 could compete with Stx2 to bind to Gb3. Our data showed that, when POS1 was co-incubated with Stx2, it reduced the interaction of Stx2 with Gb3 coated in the wells of the 96-well plate. This reduction was also dose-dependent for POS1. An average of 22.1%, 29.2% and 38.2% reduction was achieved with POS1 at 0.02, 0.2 and 1 mg/ml, respectively. POS1 could also reduce the interaction between Stx2 and Gb3 dose-dependently after pre-incubation with Stx2 at room temperature for 1 h, although at lower levels (Supplement 4). This result suggests that POS1 could not only compete with the Stx2 B-subunit to bind to Gb3; it might also damage Stx2 in some way to reduce its cytotoxicity.

4. Conclusion

The utilization of oligosaccharides derived from agricultural by-products to selectively stimulate the growth of beneficial bacteria and inhibit bacterial attachment of pathogens has proven successful for a number of pectic oligosaccharide *in vitro*. Our investigation suggests that different pectic oligosaccharide compositions, based on the extraction method, origin, molecular weight and degree of esterification, exhibit consistent *in vitro* prebiotic activity. This investigation reaffirmed two bioactivity structure-function relationships that arabinose-rich rhamnogalacturonic acids are responsible for *in vitro* prebiotic activity and oligogalacturonic acids are responsible for STEC anti-adhesion activity. Our results report, for the first time, that pectic oligosaccharides select for lactobacilli, as well as bifidobacteria, and that low molecular weight deesterified structures enhance STEC anti-adhesive activity. Before any claims for POSs to be functional food ingredients can be made, more study is needed and their efficacy in human volunteer trials must also be established. These oligosaccharides have the potential, in the near future, to join the arsenal of drugs for the therapy of bacterial diseases and health-promoting bioactive food ingredients.

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Figure legend

Fig. 1. (a) Primer and TaqMan probe locations in human 28S rRNA (GenBank accession # NR_003287) for dual primer extension and RT-qPCR amplification. Refer to Supplement 2 for primer (P) and TaqMan (T) probe numbers, names and sequences. (b) Dual primer extension assay to measure 28S rRNA depurination by Stx2 in HT29 cells. Stx2, Stx2-treated HT29 cells; Ctr, untreated cells. (c) Development of the novel TaqMan-based RT-qPCR analysis for 28S rRNA depurination measurement. The primers and TaqMan probes designed for the SRL, SRLm and 28S gene targets were validated by analyzing the 10-fold dilutions of the RT product of Stx2-treated HT29 cells in RT-qPCR reactions. The standard curves of these three gene target amplifications are shown with the amplification equations and R^2 values.

Table 1

Chemical characterization of orange pectic oligosaccharides.

Orange POS	POS1	POS2	MCP1	MCP2	OpPOS
Molecular weight (MW ×10^3)	72.8	811	9.2	17.7	140.3
Monosaccharide (mole %)					
Glucose	2.07	3.76	2.17	2.26	5.77
Arabinose	3.24	33.7	3.28	4.76	44.2
Galactose	11.6	6.85	10.3	19.2	20.2
Xylose	1.01	2.04	1.45	1.17	2.69
Rhamnose	3.69	3.47	3.53	4.29	3.56
Fucose	0.12	0.31	0.13	0.24	0.23
Glucuronic acid	0.28	0.66	0.11	0.16	0.46
Galacturonic acid	78.0	49.2	79.0	68.0	22.8
GalA:Rha	21.1	14.2	22.4	15.8	6.42
Ara:Rha	0.88	9.71	0.93	1.11	12.4
Gal:Rha	3.14	1.97	2.92	4.47	5.68
Average % degree of esterification	40.1± 0.88	42.0± 0.61	5.3± 0.52	3.3± 0.14	66.3± 0.2

Table 2

Concentration of organic acids in the fermentation samples obtained from the pH- and temperature-controlled stirred-batch fermentations, using POS 1, POS 2, MCP1 and inulin as carbon sources.

Time	Acetate				Propionate			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	2.4±1.3	1.9±1.3	2.4±1.7	1.6±1.9	0±0.1	0±0.1	0.1±0.1	0.3±0.4
10h	48.1±28.6	50.1±18.3	52.0±15.6	49.6±15.9	7.7±3.8	9.9±3.6	8.2±2.0	10.0±9.0
24h	62.2±18.4	71.4±17.9	68.4±9.9	68.7±19.1	10.5±2.2	15.1±4.1	11.6±1.8	20.4±12.1
36h	68.1±15.7	76.7±18.4	77.8±12.9	73.1±20.9	12.1±2.1	16.9±3.8	13.4±4.3	24.6±18.1
48h	68.8±18.0	83.6±22.8	82.0±14.5	68.7±20.5	12.6±1.9	18.7±5.0	14.9±4.4	22.3±15.8
Time	Lactate				Formate			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	0.5±0.5	2.0±2.0	0.5±0.5	0.4±0.6	3.2±0.8	7.2±0.8	2.8±0.5	0.2±0.5
10h	3.2±2.1	3.4±4.4	3.2±2.5	6.1±5.0	5.3±5.5	6.1±6.3	5.9±3.6	2.5±2.9

24h	0.0	6.3±1.9	0.8±0.4	0.0	0.0	0.0	0.0	0.0
36h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Time	Butyrate				Total organic acids			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	0.1±0.1	0.0±0.1	0.1±0.1	0.3±0.4	5.7±2.9	4.9±2.4	5.8±2.4	4.6±2.6
10h	2.2±3.1	3.5±2.9	3.6±3.1	5.8±5.7	66.6±37.7	73.0±26.5	72.9±20.5	74.0±20.2
24h	5.3±3.3^{b}	6.2±4.9^{b}	4.4±3.0^{b}	11.9±4.4^{a}	78.0±24.2	94.0±16.0	84.5±7.3	101±16.5
36h	6.2±3.9^{b}	8.3±4.9^{ab}	5.5±2.4^{b}	12.4±3.1^{a}	86.5±19.7	102±24.0	96.7±13.7	110±26.7
48h	7.0±4.0	10.6±6.4	6.5±3.1	12.9±2.8	88.4±21.6	113±30.6	103±16.5	103±24.1

All numbers are means of five samples ±SD, expressed as mmol/ml. Alphabetical superscript: significantly different among treatments at the same time point. Values in the same row not sharing the same superscript are significantly different ($P \leq 0.05$).

Table 3

Bacterial concentrations of the samples obtained from the pH- and temperature-controlled stirred-batch fermentations using POS 1, POS 2, MCP1 and inulin as carbon sources (Bif164: *Bifidobacterium*, Erec 482: *Eubacterium rectale/Clostridium coccoides*, Lab158: *Lactobacillus/Enterococcus*, Bac303: *Bacteroides/Prevotella*, Ato291: *Atopobium* cluster, Chis150: *Clostridium histolyticum*).

Time	Bif164				Erec482			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	7.83±0.24	7.81±0.13	7.78±0.19	7.70±0.28	8.05±0.11	8.05±0.10	8.02±0.18	8.08±0.17
10h	8.11±0.19^{b*}	8.67±0.16^{a*}	8.24±0.27^{b*}	8.66±0.24^{a*}	8.30±0.34	8.23±0.33	8.24±0.26*	8.23±0.31
24h	8.10±0.16^{b*}	8.68±0.17^{a*}	8.18±0.12^{b*}	8.82±0.29^{a*}	8.20±0.27	8.30±0.30	8.34±0.13*	8.31±0.17*
36h	8.13±0.19^{c*}	8.71±0.11^{b*}	8.13±0.13^{c*}	9.01±0.30^{a*}	8.15±0.19	8.18±0.34	8.34±0.12*	8.22±0.15
48h	8.03±0.6^{c}	8.67±0.16^{b*}	8.21±0.17^{c*}	9.09±0.16^{a*}	8.03±0.15	8.08±0.31	8.31±0.17	8.02±0.20
Time	Lab158				Bac303			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	6.40±0.19	6.40±0.23	6.50±0.19	6.44±0.15	8.04±0.22	7.96±0.24	8.09±0.26	7.98±0.31

10h	6.58±0.26*	6.90±0.20*	6.94±0.37	6.82±0.35*	8.69±0.43*	8.80±0.21*	8.85±0.40*	8.62±0.46*
24h	6.62±0.25^{b*}	7.26±0.28^{a*}	7.04±0.54^{ab}	7.28±0.60^{a*}	9.05±0.35*	9.08±0.33*	9.04±0.23*	9.05±0.24*
36h	6.55±0.54^{b}	7.11±0.57^{a*}	6.79±0.45^{ab}	7.03±0.51^{a*}	8.82±0.35^{ab*}	8.89±0.15^{ab*}	8.90±0.24^{a*}	8.88±0.26^{ab*}
48h	6.41±0.38^{b}	6.96±0.35^{a*}	6.64±0.41^{b}	7.06±0.64^{a}	8.69±0.28*	8.79±0.18*	8.67±0.32*	8.65±0.38*
Time	Ato291				Chis150			
	POS 1	POS 2	MCP1	Inulin	POS 1	POS 2	MCP1	Inulin
0h	7.46±0.17	7.43±0.19	7.48±0.16	7.57±0.18	6.07±0.34	6.09±0.39	6.09±0.30	6.07±0.22
10h	7.99±0.60	7.83±0.20*	8.21±0.44*	8.30±0.27*	6.31±0.40^{b}	6.85±0.30^{a*}	6.48±0.19^{b*}	6.35±0.17^{b*}
24h	8.00±0.54	7.95±0.32*	8.32±0.57*	8.37±0.42*	6.46±0.38	6.68±0.27*	6.44±0.32*	6.41±0.34
36h	8.10±0.49	7.98±0.32*	8.36±0.60*	8.33±0.34*	6.11±0.24^{b}	6.40±0.38^{ab}	6.61±0.47^{a*}	6.10±0.29^{b}
48h	8.05±0.38*	7.71±0.39	8.27±0.59*	8.15±0.26*	5.87±0.38	6.16±0.41	6.14±0.54	6.06±0.38

All numbers are means of five samples±SD expressed as Log$_{10}$cells/ml.

*: significant increase from 0h; Alphabetical superscript: significantly different among treatments at the same time point.

Values in the same row not sharing the same superscript are significantly different ($P\leq0.05$).

Table 4

Anti-adhesion activity of POS samples at different concentrations against *E. coli* O157:H7 strain ATCC43895 compared to untreated control sample.

	Adhesion Relative to Control (%)				
mg/mL	POS1	POS2	OpPOS	MCP1	MCP2
0.001	32.9±2.0^{a1}	68.02±2^{a3}	30.9±1.2^{a1}	39.6±1.4^{a2}	39.1±1.4^{a2}
0.005	13.7±1.5^{b1}	47.1±1.5^{b3}	8.6±0.6^{b1}	39.1±0.7^{a2}	35.0±0.7^{a2}
0.01	17.4±2.3^{b1}	51.5±3^{b3}	15.8±0.5^{c1}	40.0±1.4^{a2}	38.8±7.8^{a2}
0.05	26.0±0.6^{c1}	77.6±2^{c3}	20.9±1^{c1}	47.8±4.2^{b2}	40.8±5.7^{a2}
0.1	33.3±0.3^{c1}	79.0±0.6^{c3}	34.5±1.5^{ad1}	51.8±2.8^{b2}	44.1±7.1^{a2}
0.5	40.2±0.3^{d1}	94.8±0.2^{d4}	83.5±1.4^{e3}	55.1±0.7^{b2}	52.0±2.8^{b2}
0.8	51.1±0^{e2}	98.8±0.2^{d4}	93.5±2^{f4}	61.6±2.8^{c3}	37.0±0^{a1}
1	56.6±0.2^{e1}	100±0.4^{d2}	97.1±1^{f2}	57.3±1.8^{bc1}	57.0±1.1^{b1}
2.5	74.0±2^{f2}	100±0.8^{d3}	100±0.3^{f3}	63.5±2.8^{c1}	77.1±1.3^{c2}
5	91.3±1^{g1}	100±0.4^{d2}	100±1.8^{f2}	100±0^{d2}	100±0^{d2}

^{a,b,c,bc,d,e,f,g} indicate significant differences in inhibition of *E. coli* O157:H7 ATCC43895 adhesion relative to the control at different concentrations of the respective samples. ^{1,2,3,4} indicate significant difference in anti-adhesive activity across all the oligosaccharides at one particular concentration based on ANOVA statistical analysis ($P<0.05$). All values are the means ± standard deviation of results obtained with triplicates.

Table 5

Reduction of Stx2 depurination activity by citrus pectic oligosaccharides in HT29 cells.

Treatment	Average percentage reduction of SRLm/SRL ratio compared to Stx2-treated cells*	Standard Deviation*
POS1 1 µg/ml PC	0	0
POS1 10 µg/ml PC	3.43	0.64
POS1 100 µg/ml PC	6.59	0.18
POS1 1 µg/ml CC	13.1	5.10
POS1 10 µg/ml CC	37.7	2.45
POS1 100 µg/ml CC	44.1	2.50
MCP2 1 µg/ml PC	0	0
MCP2 10 µg/ml PC	0	0
MCP2 100 µg/ml PC	0	0
MCP2 1 µg/ml CC	13.0	0.17
MCP2 10 µg/ml CC	25.8	0.40
MCP2 100 µg/ml CC	9.51	2.08
POS2 1 µg/ml PC	0	0
POS2 10 µg/ml PC	0	0

POS2 100 µg/ml PC	0	0
POS2 1 µg/ml CC	0	0
POS2 10 µg/ml CC	20.8	5.03
POS2 100 µg/ml CC	24.2	3.74
OpPOS 1 µg/ml PC	0	0
OpPOS 10 µg/ml PC	0	0
OpPOS 100 µg/ml PC	0	0
OpPOS 1 µg/ml CC	27.9	1.59
OpPOS 10 µg/ml CC	32.6	4.43
OpPOS 100 µg/ml CC	31.3	6.32

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[*]: The average percentage reduction and standard deviation were calculated from the SRLm/SRL ratios of each treatment sample compared to Stx2-treated cells in three independent experiments.

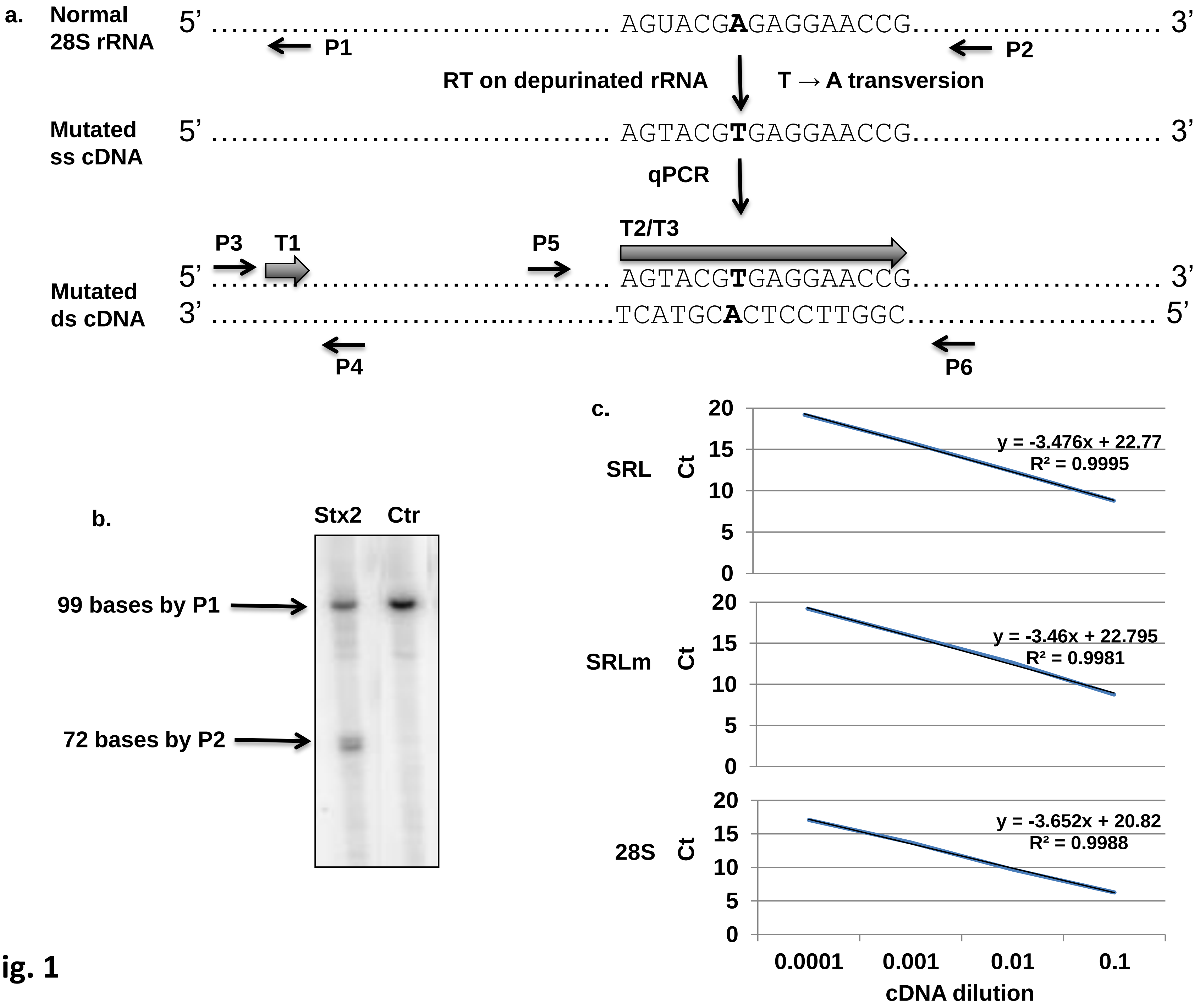
a.
Normal 28S rRNA
5' .... AGUACGAGAGGAACCG .... 3'
P1
P2
RT on depurinated rRNA
T → A transversion
Mutated ss cDNA
5' .... AGTACGTGAGGAACCG .... 3'
qPCR
P3
T1
P5
T2/T3
Mutated ds cDNA
5' .... AGTACGTGAGGAACCG .... 3'
3' .... TCATGCACTCCTTGGC .... 5'
P4
P6
b.
Stx2
Ctr
99 bases by P1
72 bases by P2
c.
SRL
Ct
y = -3.476x + 22.77
R^2 = 0.9995
SRLm
Ct
y = -3.46x + 22.795
R^2 = 0.9981
28S
Ct
y = -3.652x + 20.82
R^2 = 0.9988
20
15
10
5
0
0.0001
0.001
0.01
0.1
cDNA dilution

Fig. 1

Highlights

- Bifidogenic citrus pectic oligosaccharide (POS) structural diversity was determined.
- Five citrus pectic oligosaccharides were anti-adhesive for Shiga toxin (Stx)-producing *E. coli* O157:H7 binding to human HT29 cells.
- A novel TaqMan-based RT-qPCR assay was developed to measure the human rRNA depurination caused by Stx2.
- Citrus POS samples reduce the cytotoxicity of Stx2 holotoxin in HT29 cells.