EVALUATING STUCTURALLY DIFFERENT PECTIC OLIGOSACCHARIDES IN
INHIBITING ADHESION OF *E.COLI* O157:H7 TO HUMAN GUT EPITHELIAL
CELLS *IN VITRO*

BY

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ABSTRACT OF THE THESIS

EVALUATING STUCTURALLY DIFFERENT PECTIC OLIGOSACCHARIDES IN INHIBITING ADHESION OF *E.COLI* O157:H7 TO HUMAN GUT EPITHELIAL CELLS *IN VITRO*

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Bacterial adhesion to glycosylated cellular surfaces is a major concern in human health and disease. Inhibition of bacterial adhesion by suitable carbohydrates may lead to an anti-adhesion therapy as a novel prophylactic approach against bacterial infections and a potential alternative to the use of antibiotics. Selections of six pectic oligosaccharides derived from citrus peel albedo, which were different in terms of their monosaccharide composition and physical properties, were evaluated for their ability to interfere with the adhesion of *Escherichia coli* O157:H7 to HT29 cells *in vitro*. Attachment was determined in the human HT29 cell line by viable count of adherent bacteria. Most of the pectic oligosaccharides in buffer at pH 7.2 were anti-adhesive at a dose of 0.001 - 0.05 mg/ml, reducing adhesion of *E.coli* by 50 - 90% and concentrations of 0.5 - 5 mg/ml resulted in less than 50% reduction of adhesion to no effect. Based on the results, lower concentrations were more effective in reducing adhesion when compared to the higher concentrations. The pectic oligosaccharides with a homogalacturonan structure, low molecular weight and lower degree of esterification were the most effective in reducing the adhesion when compared to the oligosaccharides with an arabinose rich rhamnogalacturonan structure with higher molecular weight and higher degree of esterification. These results show that the pectic oligosaccharides with different monosaccharide composition and physical properties can display a wide range of anti-adhesive activity.

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1. INTRODUCTION

1.1. Food Borne Diseases / Illnesses

Foodborne diseases remain responsible for high levels of morbidity and mortality in the general population, but particularly for age-risk groups, such as infants and young, children, the elderly and the immunocompromised. The Centers for Disease Control and Prevention (CDC) estimates that 76 million cases of food-borne illnesses occur annually in the United States [1]. Preliminary data from FoodNet, a collaborative program comprised of 10 state health departments, listed the most common pathogens for food-borne illnesses from highest to lowest incidence, as *Salmonella*, *Campylobacter, Shigella, Cryptosporidium, STEC* O157, *STEC non*-O157, *Vibrio, Listeria, Yersina, and Cyclospora* [2].

Escherichia coli is one of the major causes of food poisoning worldwide. Enteropathogenic *E.coli* (EPEC) strains are common causes of *E.coli* infection, and Verotoxigenic (VTEC) infections are the most severe, with the highest lethality[3,4].

Escherichia coli is found regularly in the faeces of healthy cattle, and is transmitted to humans through contaminated food, water, and direct contact with infected people or animals. Human infection is associated with a wide range of clinical illness, including asymptomatic shedding, non-bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome, and death.

The most important virulence characteristics of *E.coli* O157:H7 is its ability to produce shiga-like toxins, the locus of enterocyte effacement (LEE) which contains genes or an adhesion molecule and other factors important in production of attaching-effacing lesions [5]. Bacterial adhesion to human cells is a key step in initiating the infection that may

lead to the development of diseases. Cell-surface carbohydrates mediate host-bacterial recognition during this adhesion through single or multiple interactions [6-7].

1.2. Limitation of Current Treatment

Bacteria assume great resistance to clearance by normal cleansing mechanisms, killing by normal immune factors, bacteriolytic enzymes and antibiotics, more over antibiotic treatment of infections can be problematic since destruction of the bacteria does not reduce toxic effects. Much of the toxin remains associated to the bacterial surface and cell lysis may actually increase free shiga-like toxin (Stx) levels available for systematic absorption in the gut lumen[8-9]

Physicians can help prevent *E. coli* O157 infections by counseling patients about the hazards of consuming undercooked ground meat or unpasteurized milk products and juices, and about the importance of hand washing to prevent the spread of diarrhoeal illness. However there is a need for an alternative prophylactic approach to antibiotic therapy apart from the preventive measures which can be taken.

1.3. Potential Alternate Treatment

Disruption of adhesive events either before or after attachment of bacteria to host tissues will interfere with colonization as long as the pathogen has not been internalized by the host cells. Effective binding of bacteria to cells requires multiple points of attachment and the adsorption of oligosaccharides could possibly prevent the bacteria from establishing multiple bonds by saturation of cell receptor sites. Competitive inhibition of the target interactions using anti-adhesive agents is therefore a rational approach toward pathogen control, representing an alternative approach to antibiotic therapy [10-11].

Added advantage to delve deeper into this approach of anti-adhesive agents is that, bacteria can adapt to many deleterious agents (i.e. antibiotic or antimicrobials) either by mutation, by acquisition of new genetic material etc. However these anti-adhesive agents do not act by killing or arresting growth of the pathogens, therefore it is reasonable to assume that spread of bacteria resistant anti-adhesive agent is expected to occur at significantly lower frequencies[8].

There is currently considerable interest in developing dietary methods using an approach more prophylactic to control food borne illnesses by means of incorporating anti-adhesives into the diet. This approach would require efficacious oligosaccharides that are available in large quantities and low cost. Production from waste material of the agricultural and food processing industries is therefore a great option [12].

2. Technical Background

2.1. Non-Digestible Dietary Carbohydrates

One approach of Non-digestible dietary carbohydrates to obtain health benefits which is widely understood as the concept of prebiotics was initially defined by Gibson & Roberfroid as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon". Since then the definition has been refined by Gibson *et al.* and prebiotics are now defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health" [13-14]. The selectivity of these dietary carbohydrates may be affected by characteristics such as the type of glycosidic linkage, degree of branching and degree of polymerization (DP), being the number of repeat monomer units in a polymer chain. The DP influences where in the large intestine fermentation occurs. Non-digestible carbohydrates with a low DP reach the proximal colon, where substrate availability and bacterial growth is generally high and the pH is low (5-6) as a result of intense acid production. In contrast, carbohydrates with a higher DP e.g. inulin may be available for fermentation in the distal colon [15-17].

Figure 1: Schematic presentation of cross-feeding in relation to microbial degradation of complex carbohydrates in the large intestine [61]
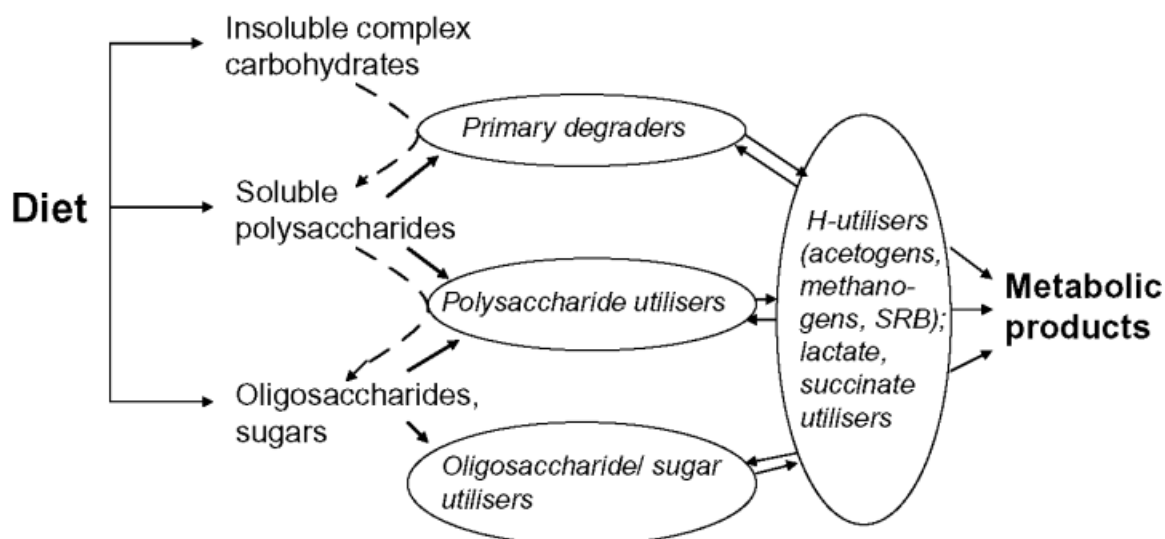
The indigestibility of ND dietary carbohydrates is a result of the ß-configuration of the glycosidic bound between monosaccharides, whereas human gastrointestinal digestive enzymes are specific for α-glycosidic bounds [18-19]. However, ND dietary carbohydrates with α-configuration also exists e.g. polydextrose and pectins. In principle, these can be degraded by human digestive enzymes, but reach the colon largely undigested due to their high molecular weight [20, 21]. Apart from the very well investigated concept of Prebiotics, there are other health benefits of ND dietary carbohydrates which have been reported and are currently under extensive investigation.

2.2. Health Benefits of Non - Digestible Dietary Carbohydrates

ND dietary carbohydrates have the potential to modulate intestinal bacterial fermentation patterns, which may in turn affect several physiological functions [22]. A large number of health-promoting effects of ND dietary carbohydrates have been hypothesized (Table 1).

Table 1: Potential health-benefits of non-digestible dietary carbohydrates [62]

Potential health-benefits of non-digestible dietary carbohydrates	References
Prevention of diarrhoea (traveller's and antibiotic-associated)	[23,24]
Treatment of inflammatory bowel diseases	[25,26]
Prevention of allergic disorders	[27,28]
Immune modulation	[29,30]
Improved mineral absorption (mainly Ca and Mg)	[31-33]
Regulation of lipid metabolism	[34,35]
Improved bowel habit	[36,37-39]
Reduced risk of colon cancer development	[40-43]

Apart from the benefits of ND dietary carbohydrates listed above a number of *in vivo* studies have investigated the potential of ND Dietary fibers on prevention of *Salmonella* infections in rodents [44, 45, 46-52]. These carbohydrates have shown to have potential to protect against pathogen adhesion and invasion by receptor mimicry [53, 54]. Attachment to epithelial cell surface receptors is often the first step in the pathogenesis of entero-pathogens and ND dietary carbohydrates acting as receptor analogues might inhibit infection, with pathogen binding to soluble oligosaccharides rather than to host cell receptors [55-57]. For example, Galacto oligosaccharide (GOS) have been shown *in vitro* to reduce adherence of Enteropathogenic *E. coli* (EPEC) to HEp-2 and Caco-2 cells, and the anti-adhesive activity of GOS was more effective than of both FOS and inulin [57]. Similarly, GOS was found to reduce the invasion of *S. Typhimurium* SL1344 and LT2 to HT29 cells lines [50]. Furthermore, pectin and pectic oligosaccharides reduced the activity of *E. coli* O157:H7 produced shiga toxin, likely by inhibiting binding of the toxin [58].

2.3.Bacterial Adhesion

In order for *E. coli* for example to cause diarrhea or other pathogenic conditions, the pathogenic bacterium must first adhere to intestinal epithelial cells and colonize the surface in order to produce effective concentrations of toxins that bind to specific receptors. Adhesion is mediated by lectin-carbohydrate interactions between bacterial fimbriae and epithelial cell surface receptors. This adhesion leads to the pathogenic conditions followed by disease. This adhesion of the bacteria to the host receptor which leads to illness or disease can be defined as bacterial adhesion.

2.3.1. Anti - adhesive activity

The ability of oligosaccharides or equivalent carbohydrate structures to inhibit the bacterial adhesion to host epithelial cells either by receptor mimicry or by saturating the receptors present on the host cells can be defined as anti-adhesive activity.

Table 2: Examples of Oligosaccharides displaying Anti-adhesive activity

Oligosaccharides	Reference
Oligofructose and inulin displayed protective action against *Listeria monocytogenes* and *Salmonella typhimirium* as well as chemically induced tumors	[59]
Inulin reduced incidence of travelers' diarrhoea	[60]
Inulin in an oral electrolyte solution accelerated beneficial bacteria and recovery from diarrhoea	[62]
Caseinoglycomacropeptide inhibited the adhesion of EPEC and VTEC strains to HT29 cells	[65]
Bifidobacterium breve plus transgalactosylated oligosaccharides inhibited *Salmonella enteritica*	[64]
VTEC strains reported to be inhibited by mannose-containing oligosaccharides	[66]
Carrot water-soluble polysaccharides displayed ability to block adherence of *E.coli* cells to	[67]

human uroepithelial cells.	
Pectin like acidic polysaccharide from the root of Panax ginseng exerted a selective anti-adhesive effect against pathogenic bacteria *Actino bacillus actinomycetemcomitans, Propionibacterium acnes, and Staphylococcus aureus*	[68]
Pectin and pectic oligosaccharides reduced the activity of three verotoxigenic *Escherichia coli* strains and three strains of enteropathogenic *E. coli*. The anti-adhesive effect was tested against verotoxins as well	[12]

The idea of combining prebiotic properties of Non-Digestible carbohydrates with anti-adhesive activities is currently under investigation. This would add major functionality to the approach of altering gut pathogenesis. To develop such efficacious oligosaccharides knowledge of the host receptors for the various bacterial pathogens is essential.

2.3.2. Bacterial Host - Receptors

Many intestinal pathogens utilize monosaccharides or short oligosaccharide sequences as receptors and knowledge of these receptor sites has relevance for developing or modeling desired oligosaccahride structures.

Table 3: Pathogen oligosaccharide receptor specificities [85]

E. coli, *Salmonella* sp. (Type 1-fimbriated)	Manα(1→2)Man, Manα(1→3)Man
E.coli (S-fimbriated)	NeuNAcα(2→3)Galβ(1→3)GalNAc
E.coli (P-fimbriated)	Galα(1→4)Gal
Salmonella typhimurium	Galβ(1→3)GalNAc

Helix pylori	NeuNAcα(2→3)Galβ(1→4)Glc
Yersinia enterocolitica	Galβ(1→3)GalNAc
Campylobacter jejuni	Fucα(1→2)Galβ(1→4)GlcNAc
Vibrio cholerae	Fuc-
Verocytotoxin / Shiga toxin	Galα(1→4)Gal
Cholera toxin, *E. coli* heat labile toxin	Galβ(1→3)GalNAc
E. coli heat stable toxin	Fucα(1→2)Gal
Clostridium difficile Toxin A	GalNAcβ(1→3)Galβ(1→4)GlcNAc

There is potential for developing oligosaccharides which incorporate such a receptor monosaccharide or oligosaccharide sequence. These molecules should have enough anti-adhesive activity to inhibit binding of low levels of pathogens. They can therefore be thought of as 'decoy oligosaccharides'.

2.4. Potential Oligosaccharides as Anti-adhesive agents

At present, the advancement of knowledge about polysaccharides from plant cell wall and plant cell wall polysaccharide cleavage enzymes allows the development of novel oligosaccharide structures. Effectively, these polysaccharides are available in large amounts notably from food industry by-products. Therefore, the use of specific hydrolysis or enzymatic treatment conditions leads to processes for oligosaccharide productions. These oligomers based on the processing technique may have a large variety

of structures which lead to improved functionality and could become an interesting way to increase the value of plant by-products in the future.

Arabinogalactooligosaccharides, arabinoxylooligosaccharides, arabinooligosaccharides, galacturonan oligosaccharides, rhamnogalacturonan oligosaccharides and pectic oligosaccharides have been successfully experimented by this process. (69, 70–72). The below table is a snapshot of the different oligosaccharides, their chemical structures and method and source of manufacture.

Table 4: Oligosaccharides chemical structure and source and method of manufacture [62]

S.No	Carbohydrate	Chemical structure	DP	Natural source/methods of manufacture
1.	**Inulin and FOS**	D-Fructose units linked by β-2.1 bounds. Terminal α-1.2-linked D-glucose.	Inulin 2-60 FOS ~ 2-7	Onion, banana, garlic, leek and chicory root Inulin: Extraction from chicory root. FOS: Hydrolysis of chicory inulin or enzymatic synthesis
2.	GOS	D-galactose units linked by β-1.4 or β-1.6 bounds. Terminal α-1.4-bound D-glucose unit.	~2-5	Human and cow's milk. Enymatic synthesis from lactose.

3.	XOS	Xylose units linked by β-1.4 bounds	~2-4	Bamboo shoots. Produced by chemical/enzymatic treatmentof xylan-rich material
4.	Cereal β-glucan	Linear chains of D-glucose units linked by β-1.4 or β-1.3 bounds	Variable >500	Oat, barley, rye and wheat. Extraction from natural sources.
5.	Pectins	Largely composed of a backbone of α-1.4-linked galacturonic acid. Five structural groups with variation in side chains and backbone.	Variable 70-100	Plant cell walls. Commercially produced from citrus peel and apple pomace.

DP: Degree of polymerization; **FOS:** Fructo-oligosaccharide; **GOS:** Galacto-oligosaccahride; **XOS**: Xylo-oligosaccahride

Since our study is primarily focused on Pectic oligosaccharides, the next section is focused on introducing Pectin and Pectic Oligosaccharides in general.

2.4.1. Pectins

Pectins are complex polysaccharides present in plant cell walls and are mainly composed of a backbone of α-1.4-linked galacturonic acid units [73]. The pectin polysaccharides are divided into five structural classes designated homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA) and rhamnogalacturonan I (RG-I) and II (RG-II) [74]. HG is a polymer of α-1.4-linked D-galacturonic acid (GalpA) that can account for more than 60% of the pectins in the plant cell wall. The galacturonic acid units may be partly methylated at C-6 or acetylated at O-2 or O-3 (Figure 9). XGA is HG substituted with D-xylose at C-3 of the GalpA units. AGA is substituted with D-apiose at C-2 or C-3 and is

found in aquatic plants. RG-I has a backbone of repeating units of galacturonic acid and rhamnose [→α-D-GalpA-1.2-α-L-Rhap-1.4→]n with side chains of α-arabinan, ß-galactan and type-I arabinogalactan. RG-II is an even more complex structure consisting of a HG backbone (7-9 residues long) with four side-chains (designated A-D) incorporating another ten different monosaccharides into the structure [74].

Figure 2: The primary structure of homogalacturonan
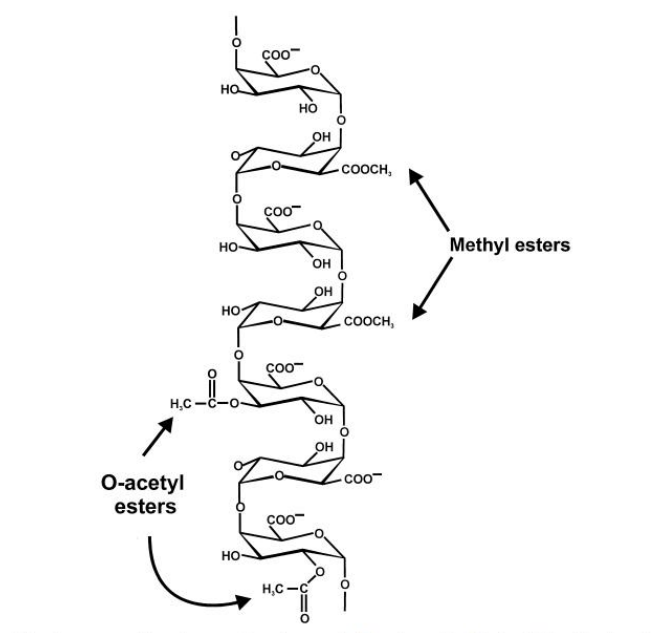
Figure 3: Major structural features of Rhamnogalacturonan I
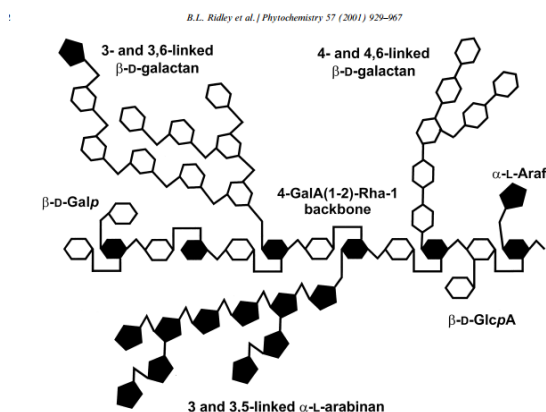
Figure 4: Major structural features of Rhamnogalacturonan II
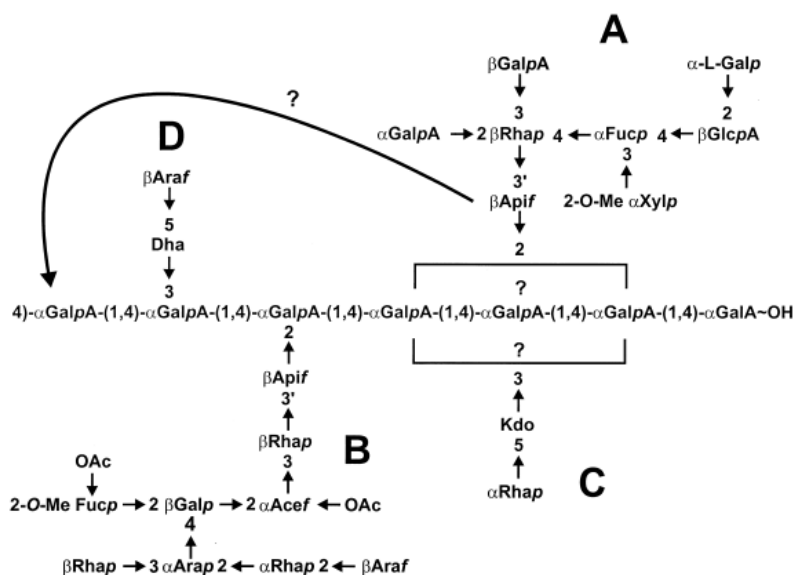
2.4.2. Pectic oligosaccharides

Pectic oligosaccharides are produced from pectin by enzymatic treatment and acid hydrolysis. (Figure 4) is the typical structure of pectic oligosaccharide: which for the most part is composed of Galacturonic acid, with few rhamnose units in between from where the arabinan and galactan units branch out. The structure is based on the source and the processing treatment.

Figure 5: Major structure of Pectic Oligosaccahride
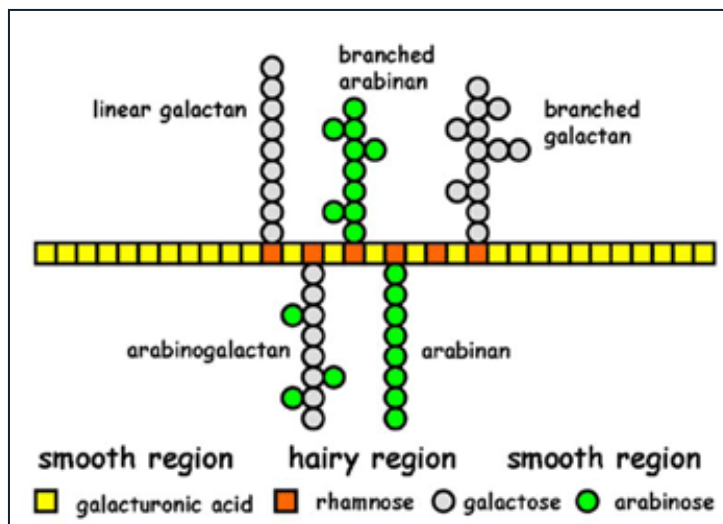
2.4.3. Oligosaccharides in food applications

Currently oligosaccharides in the form of prebiotic fibers are used as nutrition supplements and are part of many functional foods. They are used in food formulations for both nutritional and organoleptic advantages. Infant formula, soups, sauces, confectionary foods, chocolates, cakes, biscuits, meat products, fillings, beverages, yogurts and desserts are some of the product categories in which prebiotics are currently used [85]. Some of the functional properties of oligosaccharides are fat or sugar replacement, improved texture and mouth feel, fiber, foam stabilization, stability, moisture retention and heat resistance. Prebiotics/oligosaccharides not only help the growth of probiotic organisms but also has a positive effect on short chain fatty acids (SCFA) production inside the gut and also many other health benefits i.e. anti-adhesive activity. All these potential benefits collectively give oligosaccharides myriad of potential applications in food.

2.4.4. Research gaps and opportunities

Based on the literature review conducted the following gaps and opportunities are identified:

1. The extent of anti-adhesive activity of different oligosaccharides on different pathogenic bacteria were studied but the correlation between the structure of the oligosaccharides and the anti-adhesive activity is not reported

2. Anti-adhesive activity of pectic oligosaccharides (POSs) on Enteropathogenic *E.coli* have been reported; Activity on Enterohemorrhagic *E.coli* O157:H7 is not studied

3. Structural and functional relationship of different pectic oligosaccharide (POSs) samples is not studied

3.   Objectives

3.4.   Scope of the research

The previous studies that involved evaluating functionalities of different oligosaccharides showed the tremendous potential of oligosaccharides in various aspects related to health and disease. The oligosaccharides extracted from different plant sources displayed great potential in inhibiting the adhesion of various bacterial pathogens in the *in vitro* studies. pectic oligosaccharides extracted from citrus peel had displayed good anti-adhesive capabilities but the structure and the function co-relation has not been delved into. Hence the research would focus on understanding the structure and function relationship of the pectic oligosaccharide entities and extrapolating findings to future work.

3.5.   Hypothesis

Based on the previous studies, we hypothesize that pectic oligosaccharides are potential anti-adhesive candidates to serve as an alternative prophylactic approach for antibiotic therapy and there is a strong co-relation between the structure of the oligosaccharides and anti-adhesive function.

3.3. Overall Objective

In order to address the research gaps and test the hypothesis, we devised objectives to understand the co-relation between pectic oligosaccharide structure and anti-adhesive activity. Our overall objective was to evaluate anti-adhesive activity of six different pectic oligosaccharides with different monosaccharide composition at different concentrations and also co-relate the structure of the oligosaccharide to the anti-adhesive function based on the data obtained.

3.3.1. Objective

Evaluating structurally different pectic oligosaccharides in inhibiting adhesion of *E.coli* O157:H7 to HT29 cells *in vitro*

Sub-Objective 1: Evaluate the anti-adhesive effect of different pectic oligosaccharides on *E.coli* O157:H7 at different concentrations.

Sub-Objective 2: Understand the pectic oligosaccharide structure and functional relationship

3.3.2. Experimental Variables

- **Independent Variables**

  - POS samples: POS-1, POS-2, TC, HM, Orange POS, MCP

  - Sample concentrations (POS dissolved in buffer PBS) : 0.001 - 5mg/ml

  - Monosaccharide units

  - Degree of esterification

  - Molecular weight

- **Dependent Variables**

  - Anti-adhesive activity: is obtained using the formula below

$$\%\text{Bacterial adhesion of test relative to control} = \frac{\text{CFU/ml of bacteria after treatment (Test)}}{\text{CFU/ml of bacteria without treatment (Control)}} \times 100$$

4.   MATERIALS AND METHODS

4.4.   Description of materials

4.1.1. Pectic Oligosaccharides

Table5: Six Pectic Oligosaccharides used in the study

Pectic Oligosaccharides		Company
POS 1	Pectic Oligosaccharide I	EcoNugenics
POS 2	Pectic Oligosaccharide II	EcoNugenics
TC	Take Control (TC) Pectic Oligosaccahride	EcoNugenics
HM	High Molecular weight Pectic Oligosaccahride	EcoNugenics
Orange Peel POS	Pectic Oligosaccharide extracted from Orange Peel	
MCP	Modified Citrus Pectin	EcoNugenics

Orange peel POS was prepared by pilot plant-scale acid hydrolysis of orange albedo, according to Manderson et al. [75]. Pectin was precipitated from the hydrolysis with isopropyl alcohol and removed by filtration. The POS was desalted by1,000 molecular weight cutoff nanoflitration. Based on extrapolation from oligogalacturonic acid standards, the major POS peak degree of polymerization was under nine residues (1,800 molecular weight). After saponification, a minor series of peaks up to 25 galacturonic acid-equivalent residues (5,000 molecular weight) was detected in POS. These deesterified oligosaccharides were not homo-oligogalacturonic acids, based on their retention times. Orange peel POS consisted of an oligosaccharide mixture with arabinose-

rich rhamnogalacuronic acid structure. POSI, POS II, HM, TC & MCP are derived from pectin, consisting mainly of a homogalacturonan backbone that is partially methyl esterified. Homogalacturonan is interrupted periodically by regions of alternating D-galacturonic acid and L-rhamnose residues. These oligosaccharides are produced by enzymatic degradation in a continuous ultrafiltration membrane reactor.

4.1.2. High-performance anion-exchange chromatography

POS were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection, using a DX-500 (Di-onex, Sunnyvale, Calif.) system and a CarboPac PA1 column. The mobile phase consisted of a linear 5 to 375 mmol liter-1, 90-min gradient of sodium acetate in 100 mmol liter-1 NaOH. Oligogalacturonic acid standards were isolated by preparative high- performance liquid chromatography [76].

4.1.3. Reagents

Table 1: List of chemicals

Chemical	Function
Deionized water	For preparing aqueous solutions of fibers, growth media, sterile water for serial dilutions etc.
Preformulated, dehydrated tryptic soy agar medium	For preparing TSA plates to plate bacteria
Phosphate-buffered saline tablets (PBS),	~ pH: 7.0 mimicking intestinal conditions
Dulbecco's modified Eagle medium with GlutaMAX-1 (DMEM); Non-essential amino acid solution; Fetal Bovine Serum	Minimal media for reviving and enabling growth of bacteria

4.1.4. Bacterial culture

Working cultures of *E.coli* O157:H7 were prepared by inoculating the bacteria on plate count agar and incubating the agar for 18 to 24 hr at 37ºC. *E.coli* broth cultures for adhesion assays were grown in DMEM supplemented with 5% (vol/vol) fetal bovine serum and 1% (vol/vol) non-essential amino acid solution (SDMEM) and incubated anaerobically at 37C for 18-24 hrs. The overnight culture was then inoculated 1% (vol/vol) into fresh SDMEM and incubated for a further 18 to 24 hr under the same conditions. On the day of the assay, a 10% (vol/vol) inoculum was again inoculated into prewarmed SDMEM and incubated for 4 hr aerobically at 37 ºC.

4.1.5. Cell cultures

HT29 human colon epithelial cells were obtained from ATCC. Cells were grown in 25-cm^2 tissue culture flasks in SDMEM at 37ºC in 5%CO_2 until reaching confluence, split according to European Collection of Cell Cultures - recommended method and stored in aliquots in liquid nitrogen. These aliquots were used to seed 25cm^2 flasks, which after growth were split into 12-well tissue culture plates. The 12 well plates were grown to confluence before being used for the adhesion assays.

4.2. Method

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, 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. POS were dissolved in

PBS (5 mg ml-1) and sterilized by passing through 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. Control wells in which un-supplemented PBS was substituted for POS solution. All assays were performed in triplicates. The plates were swirled by hand to mix and the incubated at 37ºC aerobically for 2 hr.

After incubation, the bacterial suspension was aspirated from the wells. A 1-ml aliquot of PBS was added to each well, the plate swirled briefly by hand, and the PBS 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 37ºC 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 plate count agar plates with decimal dilutions performed in PBS as required. All plates were incubated at 37ºC for 18-24 hr before colonies were enumerated. Viable counts were calculated for all wells and the inoculum and are expressed as CFU per milliliter. 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.

Figure 6: Bacterial Adhesion Assay

Cell culture

Tissue culture flask:
HT29 cells

12 well plate

Single well

Treatment

Addition of POS

Addition of bacterial culture

12 well plate

Incubation - 2hr
(Aerobic conditions, 37° C)

Trypsinization

Serial dilution
of bacterial
suspension

Plating

Incubation

Plate Counts

$$\% \text{ Bacterial adhesion of test relative to control} = \frac{\text{CFU/ml of Bacteria after treatment Test)}}{\text{CFU/ml of Bacteria without treatment (Control)}} \times 100$$

5.   RESULTS AND DISCUSSION

5.1. Results

In the present study, five different obipektin pecticoligosaccharides and orange POS were tested for their effects on inhibiting the adhesion of pathogenic *E.coli* O157:H7 strain to intestinal HT29 cells *in vitro*. The 5 obipektin POS samples (POS1, POS2, TC, MCP and HM) were extracted using enzyme treatment rather than acid hydrolysis which was used in the manufacturing of the orange POS (Manderson et.al).

Galacturonic acid is the major ingredient of the five POSs (POS1, POS2, TC, HM, MCP) as it is the main component of Homogalacturonan, backbone of RG I and RG II [77]. Sugar composition data suggested that they have homogalacturonan structure and possibly connected to the branched RG I and small hemicellulose fragments[78, 79]. Orange POS does not have oligogalacturonic acid structure, based on their high-performance anion-exchange chromatography-pulsed amperometric detection retention times, and the galacturonic acid content is low. The Orange POS sample is composed of series of oligosaccharides of arabinose-rich rhamnogalacturonan composition. This POS is derived from the hairy region of the pectin, where rhamnogalacturonan is heavily substituted with arabinan and arabinogalactan. The large amount of glucose presented in the samples obtained was attributed to free glucose originating from the orange peel [75].

Table 7: Sugar compositions of Pecticoligosaccharide samples (%w/w of POS)

Monosaccharide (%)	POS1	POS2	TC	HM	Orange Peel POS	MCP
Glucose	2.07	3.76	2.17	9.13	48.12	2.03
Arabinose	3.24	33.7	3.28	1.67	31.19	4.29
Galactose	11.58	6.85	10.31	18.14	9.59	17.27
Xylose	1.01	2.04	1.45	4.17	2.44	1.06
Rhamnose	3.69	3.47	3.53	5.03	2.13	3.87
Fucose	0.12	0.31	0.13	0.08	0.24	0.21
Galacturonic Acid	78.02	49.22	79.03	61.7	6.29	61.24
Glucoronic Acid	0.28	0.66	0.11	0.08	-	0.14

Table 8: Molecular weight, Average % of Galacturonic acid (GA) and Degree of esterification (DE) in POS samples

Sample	Molecular Weight (Molar mass x 10^3)		GA		DE	
			Avg%	Stdev	Avg%	Stdev
POS1	72.8	1	78.02	0.20	40.1	0.88
POS2	811	6	49.22	0.56	42.0	0.61
TC	9.2	0.01	79.03	2.89	5.3	0.52
HM	109	3	61.7	0.14	40.4	0.41
Orange POS	140.3	1	21.3	0.22	66.3	0.2
MCP	17.7	2	61.24	2.76	3.3	0.14

The degree of methyl esterification in POS1, POS2 and HM is between 40% - 42%; TC and MCP between 3% - 6% and Orange POS has a degree of methyl esterification as high as 66%. POS1, POS2 and HM have higher molecular weight when compared to TC and MCP and Orange POS has the highest molecular weight. All six different pectic oligosaccharide samples were effective in inhibiting the adhesion of *E.coli* O157:H7 to HT29 cells to some degree. The anti-adhesive effect was significantly dependent on the concentrations tested. Concentrations resulting in 50 - 90% inhibition of adhesion ranged from 0.005 - 0.5 mg/ml. Less than 50% to no inhibition effect ranged from 0.8 - 5 mg/ml. POS1, TC and MCP displayed good anti-adhesive activity when compared to the other oligosaccharide candidates. TC and MCP have similar monosaccharide composition, lowest molecular weights and degree of esterification followed by POS1 which indicates that lower molecular weight oligosaccahride exhibited greater anti-adhesive activity, presumable due to increase access to receptor binding sites on the

bacteria. Similarly, a clear effect of degree of methylation also seen with low degrees

producing greater inhibition [80].

Table 9: Antiadhesive activity of POS samples against *E.coli* O157:H7 strains w.r.t control without POS at different concentrations.

S.No	Concentration (mg/ml)	POS1	POS2	TC	HM	Orange POS	MCP
1.	0.001	32.9 ± 2^a	68.02 ± 2^a	39.6 ± 1.4^a	36.7 ± 1.4^a	30.9 ± 1.2^a	39.1 ± 1.4^a
2.	0.005	13.7 ± 1.5^b	47.1 ± 1.5^b	39.1 ± 0.7^a	23.3 ± 2^b	8.6 ± 0.6^b	35 ± 0.7^a
3.	0.01	17.4 ± 2.3^c	51.5 ± 3^c	40 ± 1.4^a	26.7 ± 1.2^c	15.8 ± 0.5^c	38.8 ± 7.8^a
4.	0.05	26 ± 0.6^d	77.6 ± 2^d	47.8 ± 4.2^b	46.7 ± 0.5^d	20.9 ± 1^d	40.8 ± 5.7^a
5.	0.1	33.3 ± 0.3^d	79 ± 0.6^d	51.8 ± 2.8^b	66.7 ± 0.8^e	34.5 ± 1.5^e	44.1 ± 7.1^b
6.	0.5	40.2 ± 0.3^e	94.8 ± 0.2^e	55.1 ± 0.7^b	80.6 ± 0.2^f	83.5 ± 1.4^f	52 ± 2.8^c
7.	0.8	51.14 ± 0.6^f	98.8 ± 0.2^e	61.6 ± 2.8^c	$\sim100\pm2^g$	93.5 ± 2^g	37 ± 0^c
8.	1	56.6 ± 0.2^g	$\sim100\pm0.4^e$	57.3 ± 18^c	$\sim100\pm1.2^g$	97.1 ± 1^g	57 ± 10.6^d
9.	2.5	74 ± 2^h	$\sim100\pm0.8^e$	63.5 ± 2.8^d	$\sim100\pm1^g$	$\sim100\pm0.3^g$	77.1 ± 12.7^e
10.	5	91.3 ± 1^i	$\sim100\pm0.4^e$	$\sim100^e$	$\sim100\pm1.8^g$	$\sim100\pm0.74^g$	$\sim100^f$

[a,b,c,d,e,f,g,h,i] indicate significant difference in inhibition of adhesion of *E.coli* O157:H7 relative to control at different concentrations of the respective POS sample based on ANOVA (P<0.05) . All values mentioned are mean ± standard deviation of results obtained with triplicates

Figure 7: Antiadhesive activity of POS samples against *E.coli* O157:H7 strains, error bars indicate standard error of the mean of quadruplicate assays.
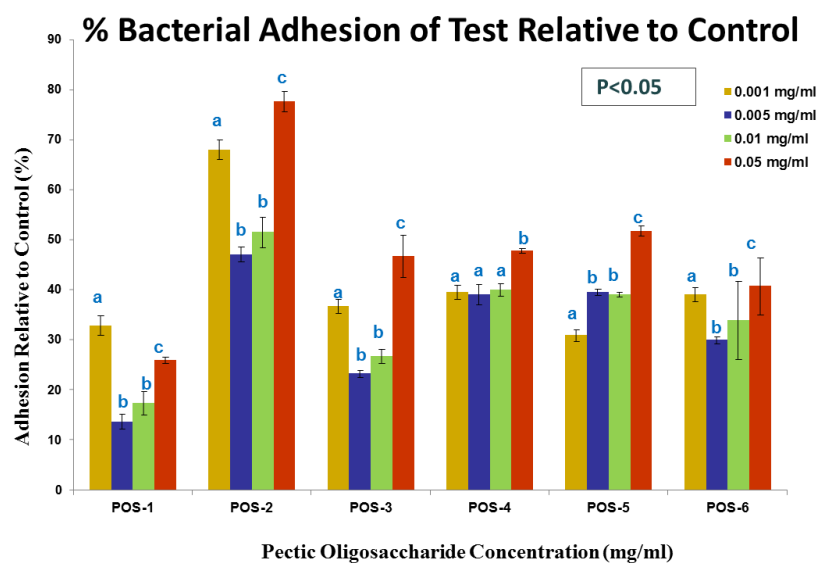
The figure above highlights the anti-adhesive activity of all the six pectic oligosaccharides at four concentrations which were most effective (0.001 - 0.05mg/ml).

Figure 8: Antiadhesive activity of POS samples against *E.coli* O157:H7 strains
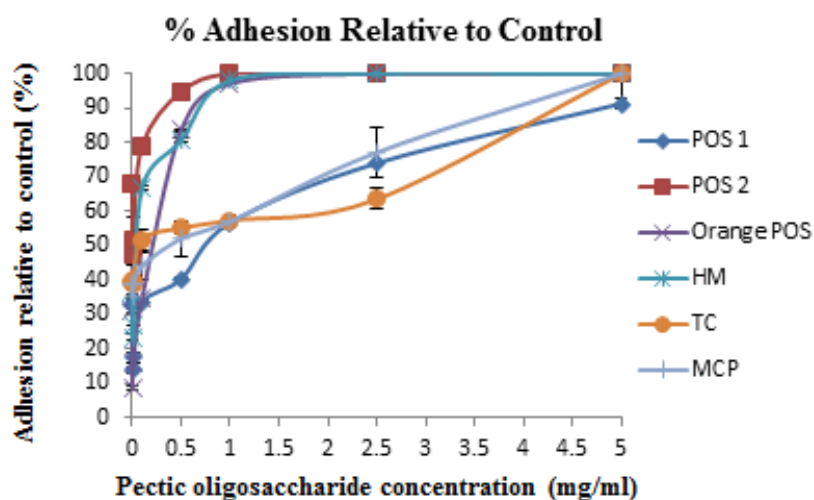
Based on the graphical representation in (Fig.8), we can see that the lower concentrations were very effective in inhibiting the bacterial adhesion and as we go to higher concentrations we see a saturation point at which we see a straight line, which implies no effect of the oligosaccharides at that concentration on bacterial adhesion. This graphical representation is a good illustration of anti-adhesive activity of oligosaccharides at different concentrations.

5.2.   Discussion

The adhesion of VTEC strains of *E.coli* is complex, involving multiple receptor mechanisms and specificities [10]. All the POSs displayed anti-adhesive activity to some degree against the *E.coli* O157:H7 strain. Although the exact mechanism is unknown, it can be assumed that the anti-adhesion is achieved due to interference of POSs at the active sites of bacteria.

It has been reported that caseinoglycomacropeptide inhibited the adhesion of EPEC and VTEC strains to HT29 cells [18]. Caseinoglycomacropeptide carries a range of sialylated oligosaccharides, which represent those present on cell surfaces [11]. In addition, VTEC strains have also been reported to be inhibited by mannose-containing oligosaccharides suggesting the involvement of type 1 fimbriae in adhesion [12, 13]. Guggenbichler et.al fractioned carrot water-soluble polysaccharides and compared them to various POS for their ability to block adherence of E.coli cells to human uroepithelial cells. They observed the most active POS were the oligogalacturonic acid disaccharide and trisaccharide[14].

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 *Actino bacillus actinomycetemcomitans, Propionibacterium acnes, and Staphylococcus aureus* while having no effects on beneficial and commensal bacteria *Lactobacillus acidophilus*, *Escherichia coli*, or *Staphylococcus epidermidis*[21].

The pecticoligosaccharide compounds POS1, POS2, HM, TC and MCP we evaluated in our study primarily compose of galacturonic acid and the Orange POS is composed of series of oligosaccharides of arabinose-rich rhamnogalacturonan composition which is not part of the receptor sequence for P-fimbriated *E. coli* strains, the minimum receptor sequence for these strains is Gal1→4Gal, a sequence not observed in our POSs. The mechanism behind the anti -adhesive effect is therefore unlikely to be one of receptor mimicry and requires further study.

Eventhough the mechanism of action of pathogenic bacteria is not very apparent; utilization of oligosaccharides to inhibit bacterial attachment has proved successful for a number of pathogens *in vitro*. Our investigation suggests that pectic oligosaccharides with different composition based on the extraction method, origin, molecular weights and degree of esterification exhibit a wide range of anti-adhesive activity. This investigation reaffirmed that oligosaccharides derived from an agricultural waste product have the potential as anti-adhesive agents. Before any claims for POSs to be functional food ingredients can be made, more study is needed and efficacy in human volunteer trials must also be established. These anti-adhesive oligosaccharides in the near future have the potential to join the arsenal of drugs for the therapy of bacterial diseases[15].

6. CONCLUSIONS

All the 6 POSs were effective in inhibiting the adhesion of *E.coli* O157:H7 to some degree

- Concentrations (mg/ml) resulting in 50 - 90% inhibition ranged from 0.001 - 0.05mg/ml and less than 50% - no inhibition ranged from 0.5 - 5mg/ml. Lower concentrations were more effective than higher concentrations

- POS-1, POS TC & MCP displayed better anti-adhesive activity compared to the other three POSs

- POS-1, POS TC and MCP with lower molecular weight & degree of esterification exhibited greater anti-adhesive activity

- POS1, POS TC & MCP with homogalacturonan structure were more effective when compared to orange POS with arabinose-rich rhamnogalacturonan structure

Overall conclusion is that all the six POSs were effective in inhibiting the bacterial adhesion to some degree (10-90%) and the structure and physical properties of POS samples seem to have played an important role. Based on *in vitro* study POSs have the potential to be an emerging alternative prophylactic approach to Antibiotic therapy.

7. FUTURE WORK

- Evaluate the effect of the six different Pectic Oligosaccharides used in the study in inhibiting the cytotoxicity of Shiga-like toxin 2 in Vero cells using RT-PCR assay

- Evaluate the anti-adhesive effect of pure Pectic Oligosaccharides structures to enable a clear understanding of the structure - function co-relation

- *In vivo* studies and human volunteer trials to confirm the data obtained in the in vitro study

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