ÉVA JERMENDI

Pectin methyl-ester distribution

2022

Pectin methyl-ester distribution; analysis, modification and biofunctionality

ÉVA JERMENDI

Propositions

1. It is meaningless to discuss the bioactivity of pectin without including a detailed characterization of this complex polysaccharide.
   (this thesis)

2. Pectin's descriptive methyl-esterification parameters must become available for all pectins.
   (this thesis)

3. It is not stress that kills us, it is our reaction to it.
   (Selye, 1976, In: Psychopathology of human adaptation, Springer, pp 137-146)

4. The availability of more resources does not guarantee better science.

5. Children who learn to grow food, will eat the food they have grown.

6. Gardening adds years to your life and life to your years.

Propositions belonging to the thesis, entitled:
Pectin methyl-ester distribution;
analysis, modification and biofunctionality

Éva Jermendi

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Pectin methyl-ester distribution; analysis, modification and biofunctionality

Éva Jermendi

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Pectin methyl-ester distribution; analysis, modification and biofunctionality

Éva Jermendi

Thesis

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“Look deep into nature, and then
you will understand everything better.”

Albert Einstein

Abstract

Pectins are complex dietary fibers having a wide range of structural features. Some of the structural characteristics can impact human health through the gastrointestinal immune system by directly binding to immune receptors and through the modulation of the intestinal microbiota. These health effects of pectin strongly depend on its precise chemical structure. The direct effects of pectin on the intestinal immune system are regulated through pattern recognition immune receptors, such as the Toll-like receptor 2 (TLR2). Furthermore, certain pectins stimulate beneficial microbiota diversity and abundance. In addition, indirect beneficial effects of pectins can occur through pectin fermentation and consequently short-chain fatty acid (SCFA) production. The aim of this thesis was to analyze the fine structure, and the immunological and fermentation abilities of citrus pectins.

Citrus pectins were studied by enzymatic fingerprinting using endo-polygalacturonase and pectin lyase to reveal the methyl-ester distribution patterns over the pectin backbone. HILIC-MS combined with HPAEC enabled the separation and identification of the diagnostic oligomers released. Novel descriptive parameters such as degree of blockiness of methyl-esterified oligomers by PG and degree of blockiness of methyl-esterified oligomers by PL provided more detailed structural information on the pectins. This approach enabled us to clearly differentiate citrus pectins with various methyl-esterification patterns. Next, the pectin structure-dependent TLR2/1 inhibition was studied *in vitro* and by using molecular docking analysis. Four structurally different pectins were studied in healthy mice for their effects on microbiota and the fermentation metabolites, such as SCFA and aryl-hydrocarbon receptor activating indole derivatives produced. It has been revealed that the degree of methyl-esterification (DM) and degree of blockiness (DB) alone were not able to explain the immunomodulation and fermentation characteristics.

Modification and characterization of pectins are valuable and necessary to further explore and optimize their bioactivity. The testing of pectins was based on their DM and DB, revealing that each pectin has its distinct immunological signature. Even the pectins having the same DM and DB could be differentiated based on their unique methyl-esterification patterns. It is now possible to identify all the fine details of pectins and relate them to their techno- and biofunctional properties.

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Chapter 1

General introduction

The project

Pectins are of interest to the food industry because of their ability to modify the physical properties of food products and they also provide health benefits. Pectins play a significant role in maintaining health, by amongst others, lowering blood cholesterol levels or increasing faecal bulking [1]. Pectins as indigestible dietary fibers are fermented in the large intestine by microbiota resulting in the production of beneficial fermentation metabolites like SCFAs [2]. Next to the indirect health effects through the microbiota and fermentation metabolites, pectins are also now known to have direct health effects through interaction with immune receptors found in the small intestine [3].

The functionalities of pectins are profoundly dependent on their highly complex chemical structure. Pectin's chemical structure may differ in sugar composition, methyl-ester level and distribution, and conformation. Detailed characterization of pectin structures is usually achieved by enzymatic degradation to form diagnostic oligosaccharides. These oligosaccharides then give information on the structure of the native pectin through chromatographic and mass spectrometric analyses.

This multidisciplinary project, "Carbs can make the difference: how pectins fuel immunity" of the Carbohydrate Competence Center (CCC) and The Dutch Research Council (NWO), is aimed to reveal the highly complex host-fiber relationships throughout the intestinal tract. As part of the project, the described research has focused on the full characterization of bioactive pectins by enzymatic fingerprinting techniques and investigating their intermediate degradation products upon fermentation. Moreover, the immunomodulatory properties of pectins were investigated and correlated to their physico-chemical characteristics.

1. Pectin

Pectin's role in plant functionality

Pectins are complex polysaccharides predominantly present in the middle lamella of the primary plant cell wall [4, 5]. As a charged polysaccharide, it affects the ion transport and porosity of plant cell walls, thereby controlling the permeability and also determining water holding capacity [4]. Moreover, pectin plays an important role in cell wall integrity, signaling networks and cell adhesion [6]. Pectin content of fruits and vegetables are higher than other

dicotyledons. Pectin properties determining the plant cell's shape, size, texture, firmness and functional properties [4].

Pectin's structural characteristics

Pectin consists mainly of the structural elements homogalacturonan (HG) and rhamnogalacturonan I (RG-I). Other structural elements are rhamnogalacturonan II (RG-II), which is a structurally very complex element, and xylogalacturonan (XGA) (Figure 1) [7]. It has been found that the relative proportions of the different structural elements vary greatly for different plant tissues, depending on both the source and the developmental stage of the plant [7, 8].

The structural element HG, also called the "smooth region," forms the backbone of pectin. HG consists of α-(1,4)-linked D-galacturonic acid (GalA) residues typically present in amounts up to 65% of the total pectin, which makes it the most prominent element in most pectins [4]. The GalA residues can be methyl-esterified at the carboxyl group at C-6 of GalA and, occasionally, can be acetylated at the O-2 and/or O-3 position of the GalA residues depending on the source of the pectin [9]. In native pectin, nearly 80% of the carboxyl groups of GalA backbone is methyl-esterified, which can decrease during ripening of fruit and vegetables due to the presences of endogenous enzymes, such as pectin methyl-esterase [10]. The percentage of methyl-esterified GalA residues within the HG backbone is defined as the degree of methyl-esterification (DM) [9]. The amount of methyl-esters over the GalA backbone is a fundamental parameter, impacting pectin's physical properties. High DM pectins are containing 50% or more methyl-esterified GalA. The GalA residues of low DM pectins are less than 50% methyl-esterified.

RG-I, also called the "hairy region of pectin", consists of a repeating unit of α-1,4-linked D-galacturonosyl-α-1,2-L-rhamnose, where rhamnose residues carry neutral sugar side chains composed of arabinans and/or galactans linked at their O-4 position [11, 12]. RG-I is often acetylated at the O-2 and/or O-3 positions of the GalA, while methyl-esterification is rare in this region [4, 13]. The RG-II consists of a homogalacturonan segment, which is branched with complex oligosaccharide side chains to which 12 different monosaccharides can be linked in a complex way [11].

XGA is a homogalacturonan polymer with BETA-D-xylopyranose substituted to part of the GalA residues. Xylosidation can vary between 25% and 75%. XGA can be partially methyl-esterified and/or acetylated [4].

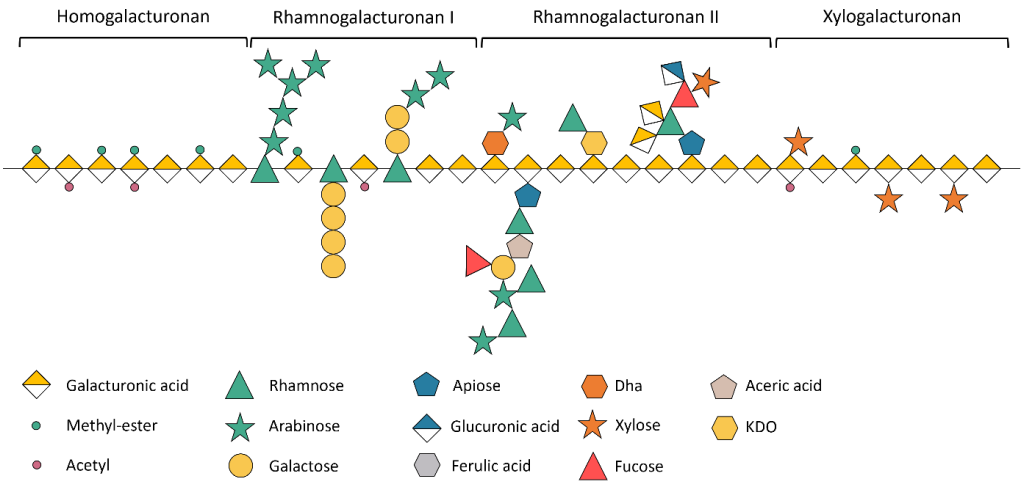

Figure 1. *Schematic representation of the pectin substructures homogalacturonan, rhamnogalacturonan I and II and xylogalacturonan. Dha: 2-keto-3-deoxy-D-lyxo-heptulosaric acid; KDO: 2-keto-3-deoxy-D-manno-octulosonic acid*

1.1. Commercial pectin

Different extraction methods impact the physico-chemical properties of pectin, like viscosity, gelling and stabilization [14]. Pectins are typically extracted under acidic conditions, and extraction conditions such as temperature, time, and pH could influence the characteristics and consequently the 'quality' of the extracted pectin [15, 16]. Acidic extraction results in the partial removal of the side chain of native pectins [16] hence commercially extracted pectins primarily consist of homogalacturonan decorated with methyl-esters and small amounts of RG-I containing few/short neutral sugar side chains [17]. The homogalacturonan structural elements of pectins mainly vary in level and substitution pattern of the methyl esters from one molecule to another.

Commercial pectins are primarily extracted from apple pomace and citrus peels [18]. As their structure strongly depend on the plant source and the extraction processes, pectin fine structure can be vastly diverse. Pectins are commonly used in food products because of their emulsifying, thickening, gelling, and stabilizing properties. GalA residues of commercial pectin can not only be methyl-esterified or acetylated, but also amidated after chemical modification, i.e. the addition of amide groups at the C-6 position of GalA residues leading to an improved gelling behavior [19]. The physical properties of commercially extracted pectins depend not only on the molecular weight, but

also on the level of methyl-esters or acetyl groups, and the distribution of these substituents over the GalA backbone [17, 20]. The level of methyl-esterified GalA residues and the distribution of methyl-esterified and non-esterified residues along the HG backbone govern most of the techno- and bio-functional properties of pectins [3, 21-23]. Low and high DM pectins hold different gelling mechanisms and behaviors.

High DM pectins are primarily able to form gels at acidic pH levels (2.6-3.4) and in the presence of more than 60% sugar content, without calcium addition [8, 18]. A low pH will decrease inter- and intramolecular charge repulsion and the high sugar content reduces the water activity and promotes chain-chain interactions [8]. The gelling behavior and speed of gel setting is determined by the DM. Ultra-rapid set pectins (DM 74-77) gel within 1-3 min after heating and subsequent cooling the solution, while the slow set pectins (DM58-65) gel within 30-120 min after having heated the solution [24].

Low DM pectins can be used in a wide range of pH (2.5-7) without sugar addition in the presence of bivalent cations, such as calcium. Low DM pectins gel by the so-called “egg box” mechanism [25], where non-esterified regions of the HG backbone are connected by calcium ions. It has been shown that non-esterified blocks of at least 8 GalA are needed for the cross-linking [8, 26]. Because of this requirement, the gel forming depends not only on the level but also on the distribution of methyl-esters over the backbone [20, 27]. Consequently, the gelling ability not only increases by decreased DM levels, but also by the more blockwise distribution of non-esterified GalA residues [20, 27]. Commercial pectins are usually tailored through chemical- or enzymatic modifications to meet specific functionalities [26].

2. Modification of pectin

Enzymes used for modification and structure elucidation of the HG backbone

As pectins are really complex polymers, the gross composition of pectin macromolecules is unable to give sufficient insights into its fine structure and consequently, its functional properties. Polysaccharide degrading enzymes are important tools to degrade pectins into smaller oligomers and study their structure [33]. Enzymatic degradation is a preferred method to analyze pectins because they are more specific to the targeted pectin molecule in comparison to chemical degradation methods. Enzymatic methods are of importance as they allow the regioselective depolymerization of pectins under mild

conditions [19]. HG is the smooth region of pectin, nonetheless, several enzymes are necessary for its degradation (Figure 2).

Carbohydrate active enzymes are classified within the Carbohydrate-Active enZYme (CAZy) database (www.cazy.org) as based on their enzymatic cleavage mechanism and amino acid sequence [28]. Furthermore, a detailed data collection on enzymes can be found in the Braunschweig enzyme database (BRENDA, http://www.brenda-enzymes.info) [29]. Within CAZy, cell-wall-polysaccharide degrading and modifying enzymes are classified in five main classes: Glycoside Hydrolases (E.C. 3.2.1), Glycosyl Transferases (E.C. 2.4), Polysaccharide Lyases (E.C. 4.2.2), Carbohydrate Esterases (E.C. 3.1.1) and Auxiliary Activities[30].

Enzymes involved in pectin degradation are Hydrolases, Lyases and Esterases. Hydrolases are cleaving the glycosidic bonds between two sugar residues while adding one water molecule. Lyases are cleaving glycosidic linkages by introducing a double bond without any water addition. Esterases catalyze the de-esterification of esters from a specific esterified substrate.

HG backbone degrading enzymes are endo- and exo-polygalacturonases (PGs) [31]. Endo-polygalacturonase (endo-PG) is able to hydrolyze the α-1,4 glycosidic bonds between two non-esterified GalA residues (Figure 2), therefore its activity is decreasing when the level of methyl-esters exceeds 40-50% [32]. Endo-PG from *Kluyveromyces fragilis* requires four consecutive non-esterified GalA residues to cut the backbone [33, 34], while e.g., endo-PG from *Aspergillus niger* needs only two neighboring non-esterified GalA residues to act [35]. Fungi, bacteria, and plants require a large variety of polygalacturonases for a complete degradation of the pectin, which demonstrates the complexity of pectin structures. *Aspergillus niger* among others, produces 7 different endo-PGs, which are each showing distinct sensitivity for methyl-esterification [36, 37]. Exo-polygalacturonase (exo-PG) hydrolyzes the oligomeric and polymeric GalA chains from the non-reducing end, and it is only active towards non-esterified GalA residues [38].

As mentioned, PGs are hindered by methyl-esters over the backbone, therefore additional enzymes are necessary for complete degradation or for pectin modification. Endo-PG will be more active hydrolyzing the GalA backbone when methyl-esters are removed by pectin methyl-esterase (PME) [39]. The mode of action of PMEs differ depending on the origin of the enzyme. The mechanism of de-esterification by plant PMEs happens in a blockwise manner, while fungal PMEs in general de-esterify the backbone in a random manner [10], although the variability between PMEs from one single fungi or plant is huge.

In addition to the hydrolytic enzymes, pectin lyases (PL) cleave the highly methyl esterified HG backbone while pectate lyases cleave the non-esterified HG backbone [40, 41]. Pectin lyases split between two methyl-esterified GalA residues by β-elimination and by that, introducing an unsaturated double bond at the nonreducing end of the GalA oligomer formed [42].

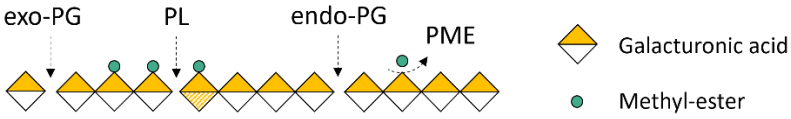

***Figure 2**. Schematic representation of pectic homogalacturonan and the enzymes needed for its modification and degradation. exo-PG: exo-polygalacturonase PL: pectin lyase, endo-PG: endo-polygalacturonase, PME: pectin methyl-esterase.*

For enzymatic fingerprinting of HG pectins endo-PG and PL degradation is a valuable method to reveal methyl-esterification patterns in pectins. Because the enzyme action depends on the methyl-esterification patterns, the released oligosaccharides reflect on those patterns and therefore are highly diagnostic. Based on the released diagnostic oligomers after the extensive enzyme digestion of citrus pectin, the different blocks, and patterns of methyl-esterification of the HG backbone can be reconstructed. For the enzymatic modification of pectin, while maintaining the high molecular weight, PME is mainly used since only methyl esters will be removed. PME is not a powerful enzyme to be used for enzymatic fingerprinting of pectin's methyl-ester distribution.

Chemical modification of pectin

Using chemical treatments, the structure of pectin can be tailored for distinctive functionality [43]. For the preparation of very highly methyl-esterified pectin, acidic methanol is used [44] to further increase the DM of pectins. In aqueous solutions the removal of methyl-esters, acetyl groups and neutral sugars may occur below pH 2, and above pH 7 at elevated temperatures [19, 45]. Alkaline conditions do not remove neutral sugars. Under these pH conditions pectin may undergo either acid or base catalyzed depolymerization of the backbone [19, 46]. To avoid the depolymerization of pectin backbone, it is important to control the temperature, therefore re- or de-esterification of pectins is usually done at 4 °C [45]. Molecular weight is an important characteristic of pectin, therefore avoiding depolymerization is advantageous.

β-elimination will only happen at pH>5-6, especially at higher temperatures. At lower temperatures, the de-esterification reaction will be relatively slow, but the β-elimination will be inhibited. β-elimination requires the presence of methyl-esters, therefore it is strongly reduced by the saponification [46]. Saponification of pectin results in a random removal of methyl-esters.

3. Pectin physicochemical properties

The properties of pectins are to a large extent determined by the HG segments within the pectin molecule [33]. Pectins are usually characterized by monosaccharide composition, galacturonic acid content, glycosidic linkages, degree- and distribution of methyl-esters, and acetyl groups and molecular weight distribution [17]. However, these characteristics do not reveal the entire fine structure of pectins.

3.1. Methods for pectin characterization

Monosaccharide composition determination

The monosaccharide composition of pectins can be analyzed after hydrolysis the polysaccharide to their composing monomers. Linkages between uronic acids within the pectin backbone are rather acid-resistant, which makes them hard to hydrolyze, therefore concentrated acids and high temperatures are applied [47, 48]. A commonly used agent for the hydrolysis of glycosidic bonds is sulfuric acid [48]. As an alternative of sulfuric acid hydrolysis, methanolysis has been defined as a rapid method for analysis of pectins resulting in high yields of both uronic acids and neutral sugars. The neutral sugars released upon hydrolysis can consequently be analyzed as alditol-acetates by using gas chromatography with flame ionization detection (GC-FID) [49], gas chromatography with mass spectrometry detection (GC-MS) [50], or directly without derivatization by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [48]. Quantification of UA is performed by the (automated) colorimetric m-hydroxydiphenyl assay [51, 52] or by HPAEC after methanolysis of soluble pectins. Methanolysis/HPAEC can distinguish between glucuronic acid and GalA, which is not possible with the m-hydroxydiphenyl assay [51, 52].

Analysis of the esterification of pectin

The methyl-esters and acetyl groups are important structural features of pectins [24]. Numerous methods have been established to quantify methyl-esters and acetyl group. The quantification methods usually involve saponification of the substituents. A traditionally used method is the titration of carboxylic acids present in a pectin solution, before and after saponification with sodium hydroxide [53]. The methyl-ester content of pectin can be also determined from the methanol released upon saponification using alcohol oxidase [54]. By using high performance liquid chromatography (HPLC) analysis the methyl and acetyl content released can be measured simultaneously [55]. Another easy and widely used method for measuring methyl-ester level DM is head-space gas chromatography (HS-GC) [56].

Analysis of glycosidic linkage types

Glycosidic linkage analysis is widely used for structural elucidation of both oligo- and poly-saccharides. It is performed by the labelling the free hydroxyl groups in the polymer and further conversion of the individual sugars to partially methylated alditol acetates, which can be analyzed by GC-MS [57]. The positions glycosidic linkages for the sugars are determined by identifying the partially methylated alditol acetates [58]. GalA has to be first reduced to galactose before being included in the linkage analysis [59].

Molecular weight distribution

To monitor the molecular weight (Mw) distribution of intact pectins and to screen the degradation of pectins into mono- and oligosaccharides after enzymatic digestion, high performance size exclusion chromatography (HPSEC) is used [60]. HPSEC shows all (polymeric) material due to its refractive index (RI) detection and allows even quantification of the degradation of pectin with e.g., enzymes [61]. HPSEC gives information on the molecular weight distribution of pectins before and after enzymatic digestion, but it does not show information on the individual oligosaccharides and their substituents. Moreover, the results depend on the hydrodynamic volume, therefore the shape of the pectin influenced by the DM and branching, and not the actual Mw. For accurate Mw measurement, calibration with structurally similar (pectin) standards with known molecular weight are needed [62].

The development of HPSEC with on-line multi-angle laser light scattering (MALLS) detectors simplified the Mw determination, as there is no need for the column calibration with Mw standards. Using MALLS, the scattered light intensity produced by the sample is measured by fixed angle detectors [63]. However, the weakness of MALLS is that pectins may form aggregates, that may dominate the light scattering detection, therefore the aggregates must be filtered out prior the analysis [63, 64].

3.2. Analytical tools for structure elucidation

Intact pectin analysis

The methyl-ester distribution patterns contribute strongly to the physical behavior of pectins. By using nuclear magnetic resonance (NMR) spectroscopy the DM and the distribution patterns of methyl-esters can be analyzed [65]. The visualization of the intact pectin molecule is really difficult because the proton signals of methyl-esterified GalA residues are overlapping, therefore it is hard to conclude on methyl-esterification of longer sequences. The complexity and the high viscosity of samples undesirably affects the resolution of NMR [66].

Fourier transform infrared spectroscopy (FT-IR) is used for the determination of DM. Depending on the absorption intensity of methyl-esters at various wave numbers in the near infrared the DM of an intact pectin or pectin containing material can be determined [67, 68]. FT-IR however has some disadvantages, particularly it's sensitivity to the presence of proteins, pH and ionic conditions [67, 68].

Preparative anion exchange chromatography has been a useful tool to differentiate various sub-populations within the pectin preparation based on their charge density [17]. However, pectin populations having a different DM but a rather similar charge density, may elute at the same ionic strength, and that makes the elution pattern interpretation difficult [17]. To get a clearer picture on the methyl-ester distribution of the pectins, fractionation based both on size and charge, and enzymatic treatment of the populations is necessary [17, 62].

Using capillary electrophoresis (CE) the DM, and molecules with a different DM values can be estimated, meaning that the DM of individual molecules are recognized by measuring the net charge [69-71]. The intermolecular methyl-ester distribution of pectin samples contains also information on the pattern of methyl-esterification [72].

Pectin oligosaccharide analysis

As mentioned above, a suitable approach to reveal methyl-ester distribution patterns is using pectin degrading enzymes which degrade the pectin backbone into diagnostic oligomers. These oligomers can be identified and quantified using different chromatographic and mass spectrometric techniques. Enzymatic fingerprinting can provide data on fine structural details, and the methyl-ester distribution patterns can be revealed. There are many analytical tools that can be used to explore the fine structures of pectins through pectic oligosaccharides (Table 1).

High performance anion exchange chromatography (HPAEC) helps to separate, identify and quantify pectic oligosaccharides [17, 33]. Unfortunately, because of the high pH (pH 12) used during the analysis methyl-esters from the oligosaccharides are removed, thereby losing key structural information [73]. To study also the methyl-esterified oligomers, HPAEC-PAD coupled with MS at pH 5 could be used [34]. However, the high salt concentration of the eluent requires desalting to make this technique compatible with the MS [34].

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is often used for a quick and easy MS analysis to identify pectic oligomers in a mixture [74]. However, no distinction can be made between isomers having the methyl-esters on different GalA residues within the oligomer and an accurate quantification of pectic oligomers is not possible with MALDI-TOF MS.

Capillary electrophoresis (CE) can be also used for analyzing pectin oligomers after enzymatic digestion. CE allows the separation oligosaccharides and the determination of blockiness [75]. Using CE, it is possible to characterize pectins for more parameters at once, such as DM and DB needing only 50 µg of pectin [75]. CE with laser-induced fluorescence (LIF) [76] requires labeling of the oligosaccharides at the reducing end using fluorescent chromophore, and a drawback is that it excludes the analysis of the non-reducing oligosaccharides. Moreover, it was found for CE-LIF that the resolution was unsatisfactory when analyzing pectin hydrolysates containing methyl- esterified oligoGalAs. CE-MS [77] can also successfully be applied to characterize and quantify oligosaccharides allowing the separation of acidic oligoGalAs without labeling/derivatization in a rather good performance [78].

Polysaccharide analysis using carbohydrate gel electrophoresis (PACE) analysis can reveal the intramolecular methyl-ester distribution of the pectin oligomers. These oligomers can be also separated and quantified [79, 80]. PACE

involves the derivation of the oligosaccharides by a fluorophore followed by separation by gel electrophoresis [79].

Moreover, by hydrophilic interaction liquid chromatography (HILIC) coupled with online electrospray ionization ion trap mass spectrometry (ESI-IT-MS) and evaporative light scattering detection (ELSD) diagnostic oligomers can be separated by DP, identified, and quantified [81].

Table 1. *Overview of analytical methods of pectic oligosaccharides, and methyl-esterification patterns. Including the pectin sources, enzymes used for pectin digestion and some drawbacks of the methods.*

Methods	Pectin source	Enzymes used	Detection	Drawback	References
HPAEC-PAD pH12	citrus pectin	endo-PG from *Kluyveromyces fragilis*	DB and DB_{abs}	High pH, loss of methyl-esters	[82-84]
HPAEC-PAD pH5	lemon pectin	endo-PG from *Kluyveromyces fragilis*	separation based on charge and DP & information on the enzyme specificity	Quantification and annotation is hampered because of high salt concentrations	[34]
MALDI-TOF MS	amidated pectin	endo-PG from *Kluyveromyces fragilis*	a fast screening of methyl-esterified oligosaccharides	quantification is not possible, no isomer-distinction	[34, 85]
MALDI-TOF-MS & ESI-MS^n	commercial and modified lime pectin	combination of endo-PG II and exopolygalacturonase (exo-PG)	determining the amount of contiguous GalA in a block	quantification is not possible	[86]
Preparative SEC & MALDI-TOF-MS & CE	modified citrus pectin	PL from *Aspergillus niger*	Quantification of methyl-esterified pectin regions. Determining DBMe and DB_{abs}Me	Fractionation is laborious	[87]
CE	lemon pectin	endo-PG II from *Aspergillus niger*	intermolecular methyl-ester distribution		[69, 71]
CE-MS & CE-LIF	pectin oligomer mixture	endo-PG from *Kluyveromyces fragilis*	Characterization and quantification of neutral oligosaccharides	labelling with a fluorescent group is required and co-elution of oligosaccharides	[77, 78]
PACE	Lemon pectin	Endo-PG from *Aspergillus japonicus*	separation, detection, and quantification of oligogalacturonides	DP and DM of needs to be known to generate the right absorbance conversion factor to obtain the concentration	[79, 80]
HILIC-ELSD/ESI-MS^n	Sugar beet pectin	Endo-PGII and PL, endo-galactanase, endo-arabinanase, RG-hydrolase, exo-arabinase, fungal PME	analyzing and quantifying partially methyl-esterified and acetylated oligomers	quantification is not reliable and reproducible	[81]
ESI-MS^n	sugar beet pectin	endo-PG, PME, endo-arabinanase and endo-galactanase	Analysis of partially methyl-esterified oligomers up to DP 10		[74]
HPAEC-ELSD	citrus pectin	endo-PG from *Aspergillus aculeatus*	separate, detect and quantify oligomers	Co-elution of oligomers	[88]
NMR	citrus pectin		Classifying distribution of methyl-esters and carboxyl groups	quantification is not possible and requires purified, homogeneous pectin samples	[65]

All these different analytical methods illustrate that pectin is a highly complex and structurally diverse polysaccharide [89]. There has been extensive research done to reveal its chemical structure, but there is still much to be discovered to correlate bio- and techno functionalities of pectins to its unique assets. As described in the table, numerous analytical techniques are available to characterize pectic oligosaccharides, however each technique has limitations for the simultaneous analysis of a wide range of oligosaccharides. The main shortcoming observed for pectin analysis is the lack of an analytical technique that can separate and characterize all GalA oligosaccharides released. During pectin analysis, structural features such as methyl-esters must be preserved. Furthermore, most analytical methods do not combine both the separation and detailed characterization of the heterogenous mixtures of pectic oligosaccharides. The analysis of mixtures of pectic oligosaccharides requires a combination of fractionation and use of multiple analytical techniques. Hence, there is a need to establish a method for explicit separation, identification and quantification of a broad range of pectic oligosaccharides.

Combination of analytical methods with enzymatic fingerprinting approaches, provide a powerful tool to elucidate the fine structure of pectin regarding the methyl-ester distribution.

3.3. Parameters to describe methyl-ester distribution

Describing non-esterified GalA blocks

Within the HG, the methyl-ester distribution is one of the most important parameters and even minor differences in the level or distribution of methyl-esters result in significant differences in physical behavior [69, 71, 90]. Pectins in general are a mixture of several populations, therefore it is important to know that the level- and distribution of methyl-esters can differ within one pectin preparation. The GalA residues in a DM60 pectin are, on average, for 60% methyl-esterified, nevertheless, pectin molecules will be present with a DM ranging from e.g., 30-80 indicating intermolecular differences. The distribution of methyl-esters within one pectin preparation will also differ due to intramolecular differences [91]. For example, two pectin molecules having both a DM of 60 may show intramolecular differences since the distribution of methyl-esters may differ largely. In commercial pectins, many molecules may have a slightly different DM, and in addition, the ones having a similar DM may have a different distribution of the methyl-esters over the GalA chain. The distribution of non-esterified GalA residues along the pectin backbone can be described as random or blockwise (Figure 2).

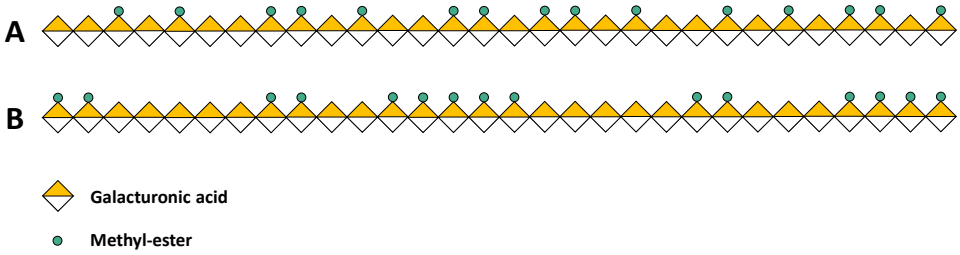

Figure 3. *Schematic representation of **A** random and **B** blockwise distribution patterns of non-esterified GalA residues in a 50% methyl-esterified pectin.*

The distribution of non-esterified GalA residues is defined by the degree of blockiness (DB) or absolute degree of blockiness (DB_{abs}) [17, 33, 39, 89]. DB is defined as the moles of GalA residues present as non-esterified mono-, di- and triGalA released by endo-polygalacturonase, related to the number of moles of non-esterified GalA residues present and expressed as percentage (Equation 1). The DB_{abs} is calculated as the moles of GalA residues present as non-esterified mono-, di- and triGalA residues released by endo-polygalacturonase expressed as percentage of the total moles of GalA residues present in the pectin molecule (Equation 2). The DB and DB_{abs} parameters do not provide information on the exact sizes of non-esterified GalA blocks [87].

Equation 1.

$$DB = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_n \text{released}]_{\text{non-esterified}} \times n}{[\text{total non-esterified GalA in the polymer}]} \times 100$$

Equation 2.

$$DB_{abs} = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_n \text{released}]_{\text{non-esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100$$

While these parameters are only focusing on the non-esterified GalA sequences, there are other descriptive parameters which are illuminating the methyl-esterified segments of the HG.

Describing methyl esterified GalA blocks

To study the methyl-esterified GalA residues of pectins, Ralet et al. [87] described the degree of blockiness of highly methyl-esterified stretches (DBMe) and absolute degree of blockiness of highly methyl-esterified stretches ($DB_{abs}Me$) based on the oligomers released upon pectin lyase (PL) digestion. In these parameters the amount of unsaturated GalA oligomers released by PL is determined. $DB_{abs}Me$ has been calculated as mole of GalA residues present as unsaturated methyl-esterified GalA per 100 mole of total GalA in the polymer released after PL digestion. Also Remoroza et al [92] included PL in their enzymatic fingerprinting to release GalA oligomers from highly methyl-esterified GalA regions. In their study, acetylated sugar beet pectins were degraded by both PG and PL. All GalA residues that were present as unsaturated GalA oligomers (DP 2–8) being methyl-esterified (and frequently acetylated) in sugar beet pectins were quantified and calculated as the degree of hydrolysis by PL (DH_{PL}) (Equation 3). DH_{PL} and DBMe are rather similar in definition although there is a difference in the enzymatic release, because of the pectin substrates used [87, 92]. Remoroza et al. [92] also introduced the degree of hydrolysis by PG (DH_{PG}) (Equation 4) to calculate the proportion of GalA residues present as saturated monomer or oligomers up to DP8 in mole per 100 mol of total GalA in the pectin polymer [41]. DH_{PG} represents both the non-esterified and also the partly methyl-esterified or acetylated GalA sequences. Figure 3 illustrates the descriptive parameters of pectin.

Equation 3

$$DH_{PL} = \frac{\sum_{n=2\text{-}8} [\text{unsaturated GalA}_n \text{ released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100$$

Equation 4

$$DH_{PG} = \frac{\sum_{n=1\text{-}8} [\text{saturated GalA}_n \text{ released}]_{\text{non-esterified and esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100$$

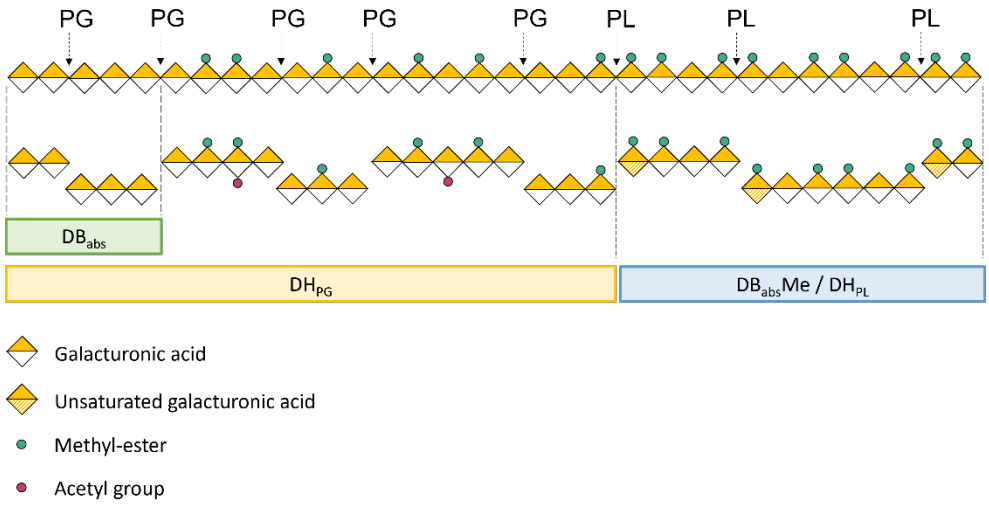

***Figure 4**. Schematic illustration of the enzymatic degradation of a hypothetical pectin and the released diagnostic oligomers represented by the descriptive parameters. DB_{abs}: absolute degree of blockiness, DH_{PG}: degree of hydrolysis by PG, DH_{PL}: degree of hydrolysis by PL, and $DB_{abs}Me$: absolute degree of blockiness of methyl-esterified residues [33, 87, 92].*

To get a full picture of the entire pectin backbone, additional studies and descriptive parameters are needed that can differentiate pectins even with seemingly similar characteristics. Ralet et al. only studied the PL degradable part of pectins, which is useful for high DM pectins [87], and Daas et al. only studied the PG degradable segments of pectins, which is highly informative for low DM pectins [33].

In general, most studies look at the DM values of pectins to judge pectin functionalities and in some cases information either on the methyl-esterified or the non-esterified segments of pectins can be found, but never both. The combined approach of degrading pectins with both PG and PL simultaneously would be highly useful to describe citrus pectins in great detail, but have not been used so far. Incubating pectins with PG and PL will allow the discovery of so far hidden structural features of citrus pectins and will explain techno- and bio-functional activities.

4. Pectin's bio-functionality

Pectin continue to gain great interests for its bioactivities, as many studies have been reported pectin's health promoting effects [2, 3]. Pectin is considered to provide various health benefits such as delayed gastric emptying [93], improved intestinal function [94], antidiabetic effects through decreased cholesterol and glucose absorption [95, 96], increased fecal mass [97, 98], antitumor, and anti-inflammatory effects [99-101]. Moreover, pectin can bind toxic (ionic) compounds as well, preventing toxicity [101-103]. Pectin is also recognized as potentially prebiotic, as it cannot be degraded by digestive enzymes, but can be fermented by the microbiota in the colon [2, 104]. Daily consumption of pectins could help the prevention of various health issues and assisting in the maintenance of good health [105]. The fermentation of pectins is determined by the size of the fibers and also on specific structural characteristics [106]. The more complex a fiber is, being highly branched with different building blocks and/or various linkage types, the more difficult fermentable it will take to be fermented by the microbiota, resulting in a beneficial fermentation along the entire length of the intestine [107]. Previous studies have also explored the structure-function relationships between pectins and pattern recognition receptors, through which pectins have shown immunomodulatory effects [3, 108].

Table 2. *Biofunctionality of pectin linked to source, mechanism, and location of action.*

Health benefit	**Type/source of pectin**	**Mechanism**	**Location of action**	**Reference**
Delayed gastric emptying	high DM pectin	Viscosity and water holding capacity	Stomach	[93, 109]
Improvement of bowel function	low DM pectin	Viscosity and fermentability	Intestine	[94, 97, 98]
Fecal bulking	low DM pectin	Viscosity and water holding capacity	Large intestine	[1]
Antidiabetic	high DM pectin	Viscosity and reduced glucose absorption	Pancreas	[95, 96, 110]
LDL cholesterol-lowering	citrus pectin	Viscosity and inhibiting bile acid absorption	Intestine	[99, 100, 105]
Cancer-preventive effects	modified citrus pectin	Binding to and inhibiting galectin-3 (GAL3)		[111, 112]
Anti-inflammatory effects	citrus and apple pectins	interaction with immune cells	Small intestine	[113]
Binding of metals and toxins in the digestive tract	citrus pectin, apple pectin, modified pectin	Ion binding	Intestine	[114-116]
Potential probiotic	low methylated pectins and pectic oligosaccharides	Fermentability	Colon	[2, 104]
Suppressing radioactive substance absorption	fruit pectin	Binding radioactive substances	Intestine	[103]
Immunomodulation	low DM lemon pectin	Binding to immune receptors, electrostatic (ionic) interaction	Small intestine	[3, 108]

Table 2 summarizes the health effects of pectins, however only the decreased cholesterol and glucose absorption health benefits are authorized by The European Food Safety Authority (EFSA) as health claim. Characteristics of pectins, such as viscosity or structural details are not mentioned by EFSA. In literature, the cholesterol lowering effect of pectins is associated with their high viscosity [98, 117]. EFSA also does not differentiate health claims of pectins, concerning details like origin, molecular weight, or DM which are highly related to techno-functional properties of pectins, hence to their bioactivity [96]. The claims and studies on pectin show that health effects credited to pectins are extensively discussed, however, the mechanisms behind the effects are frequently unknown. As mentioned above, pectins have highly diverse compositional characteristics and they are attributed to many health effects. Nevertheless, pectins used in health studies often lack structural characterization, which is a reason for many inconsistencies in the literature.

4.1. Immunomodulation

Direct effects of pectins in the small intestine

Pectins can preserve the gastrointestinal immune barrier which is the gatekeeper of the human body. The immune barrier is composed of the mucus layer and the epithelial cell layer, together they stop luminal molecules from entering the lamina propria holding immune cells [118]. Gastrointestinal immune barrier disfunction may lead to immune disorders including autoimmune and inflammatory diseases [119]. The immune barrier function is improved by increasing the consumption of dietary fibers, such as pectins. Pectins are indigestible by digestive enzymes in the small intestine therefore they can interact directly with the immune receptors in the mucus layer [108]. The possibility of pectins interacting with the immune system has been explored during the last decade. Recent studies have shown that pectins can stimulate TLRs in the intestinal epithelial layer to induce beneficiary immune effects [3, 108]. The direct binding of pectins to the TLRs was shown to be one of the main mechanisms by which pectins can improve health or prevent disease [3, 108].

Pectin binding to TLR2 can inhibit the dimerization with TLR1 receptor, or binding can also activate the TLR2 receptor. Sahasrabudhe et al. observed that pectins were able to substantially inhibit TLR2/1 dimerization. Furthermore, the same authors showed the direct binding between pectins and TLR2 and demonstrated that low DM pectins are having the highest level of inhibition and the strongest binding, and the inhibition and binding of pectins

decreased with increasing DM levels [108]. On the other hand, Vogt et al. studied both the immunomodulatory and epithelial barrier protective effects of pectins. Structural features, such as chain length and DM were analyzed to evaluate pectin's structure-response effects. They have demonstrated that higher DM pectins had a stronger TLR2 activation effect. When testing pectin oligomers they have found that pectins lose their TLR2 activating potential, meaning that an intact pectin backbone is essential for TLR2 activation [3]. These observations suggest that there may be a requirement for intact pectins and TLR2 binding both in case of the receptor inhibition and activation, however the influence of methyl-ester distribution patterns on TLR2 has remained unclear.

Pectin fermentation in the large intestine

As pectins are not digested by human digestive enzymes, they become subjects of fermentation by the large intestinal microbiota [120]. Pectin is essentially completely fermented by the large intestinal microbiota [121]. Pectin fermentation in the intestine is a multistep process: pectin degradation and formation of GalA oligomers and monomers, fermentation of the GalA monomers, and SCFA formation as well as different gases as byproducts [121]. Soluble fibers are usually faster and more completely fermented compared to insoluble fibers [122]. It has been shown previously *in vitro* that high DM pectins are more slowly degraded compared to low DM pectins [123]. In the study of Dongowski et al., it was found that next to the fast fermentation rate of low DM pectins, unsaturated oligoGalA appeared, suggesting that pectate lyases are key enzymes in pectin degradation by the microbiota [123]. Results of Jensen and Canale-Parola [124] showed that Bacteroides species (*B. pectinophilus* and *B. galacturonicus*) are pectin degraders in the human microbiota. Cell-wall-associated pectin-degrading enzymes were also found in *B. thetaiotaomicron* [125] and *Clostridium butyricum* [123], both isolated from human faeces.

Previous studies have explored the probiotic potential of pectins. In a study by Gómez et al., lemon peel and sugar beet were analyzed for their prebiotic potential *in vitro* [2]. It was shown that sugar beet pectic oligosaccharides have bifidogenic potential upon fermentation and that lemon peel pectic oligosaccharides induced lactobacilli populations [2]. These results show that pectin oligosaccharides are emerging prebiotic candidates. Pectin oligosaccharides hold functional properties in the regulation of microbiota. Previous studies have shown that pectin oligosaccharides are possibly more potential prebiotic candidates than intact pectin [2, 104, 126].

Moreover, it has been found that dietary fibers influence other fibers' fermentation, resulting in a preferred fermentation of one of the fibers in the proximal colon and shifting the fermentation of other fibers to the distal colon [127, 128]. Tian et al. have shown that supplementation of low DM pectin modulated the location of fermentation of other fibers present in the diet and had beneficial effects for gut microbiota composition and SCFA production [128].

Dietary fibers stimulate the growth of beneficial microbial families and through fermentation they also contribute to the production of short-chain fatty acids (SCFA) [120]. The most abundant SCFAs found in the intestinal tract are butyrate, propionate, and acetate [128]. Butyrate is one of the most studied SCFA associated with anti-inflammatory effects, energy expenditure and better gut motility [129].

Dietary fibers play an important role in maintaining a healthy gut as fibers serve as energy source for the microbiota [130]. Through this mechanism, pectins have indirect health effects through the stimulation of intestinal microbiota and the microbial metabolic activities [2]. Recent studies have shown that SCFA play a beneficial role in health and immunity [129, 131]. Next to the fiber fermentation products, the microbiota can produce other intermediate metabolites, such as tryptophan metabolites, which can potentially regulate intestinal immunity [132]. Pectins are proposed to promote beneficial bacteria growth such as *Lachnospiraceae* and *Bacteroidaceae* suppressing the pathogenic bacteria such as *Staphylococcus aureus* [120, 133].

To be able to relate structural data to biological effects offers foundational information for the food and pharmaceutical industry to use specific, dedicated, and tailored pectins to come to healthier food. In literature, most of pectin's beneficial effects are reported without proper structural characterization. Therefore inconsistencies can be found in literature frequently and many results are hard to compare. Pectins are typically described by their physical functions, however the structural characteristics contributing to their biological function would be more valuable.

Thesis outline

It has been already highlighted that dietary fiber characterization is crucial [98]. The main aim of the project was to elucidate the fine structure of citrus pectins. However, the current characterization and fingerprinting methods and descriptive parameters for methyl-ester distribution patterns are not yet sufficient for citrus pectins. There is a need to develop high throughput techniques for the analysis of methyl-ester distribution patterns of pectins and to use such sophisticated methods to characterize bioactive pectins and link their structure to fermentation- and immune modulating properties. In this thesis distinction between various, rather similar, pectins will be made by exploring both chemical structure, fermentability and immune responses.

In **Chapter 1**, the background of the project is presented. Information is given on the structural characteristics of native and commercial pectins including their techno- and biofunctionality regarding immunomodulation and pectin fermentation. Furthermore, a review on the various analytical methods and tools available for pectin characterization is provided.

Citrus pectins were studied by enzymatic fingerprinting using a simultaneous enzyme treatment as described in **Chapter 2**. Enzymatic fingerprinting using endo-PG and PL followed by combined HILIC-MS and HPAEC analysis revealed the methyl-ester distribution over the GalA backbone. The structural information on pectins was elucidated by quantitative parameters like DB_{PGme} and DB_{PLme}. We differentiated between citrus pectins having similar or quite distinct methyl-esterification patterns and partly reconstructed the structure of the parental pectins.

In **Chapter 3**, it is described how pectins were analyzed for their structural characteristics using enzymatic fingerprinting. We focused on pectin's anti-inflammatory effects via (Toll-like receptor) TLR binding. DM and DB dependent TLR2/1 inhibition was observed. Pectins were compared for their effects via inhibition of TLR2/1 depending on the level and the blockwise distribution of non-esterified GalA present in pectins.

The structure function relationship between commercial pectins, modified pectins with tailored methyl-ester distributions and TLR2/1 inhibition are described in **Chapter 4**. The structural characteristics of pectins were studied using the methods and descriptors as reported in **Chapter 2** in order to understand the interaction of pectins with TLR2/1. Next to TLR2/1 inhibiting properties of intact pectins, two GalA heptamers $GalA_7Me^{1,7}$ and the $GalA_7Me^{2,5}$ were used in a molecular docking analysis to study their binding mechanism

to TLR2 *in silica*. The impact of the methyl-ester distribution patterns of pectins on TLR2 were assessed.

In **Chapter 5,** the modulation of composition and activity of mice microbiota, SCFA production and AhR activation by pectins is demonstrated. Healthy mice were supplemented with structurally different lemon pectins in a dose of 3 mg/day for 1 or 4 weeks. The effect of supplementation with structurally different pectins was studied on the microbiota diversity, composition and on fermentation metabolites at different sites within the intestinal tract of mice.

In **Chapter 6**, the results obtained in this research are discussed. In addition, the conformation, and structure-function relationships of pectins are assessed in relation to immunomodulation and fermentation. Future perspectives regarding pectin's health benefits are discussed.

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Chapter 2

Revealing methyl-esterification patterns of pectins by enzymatic fingerprinting: beyond the degree of blockiness

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Abstract

Citrus pectins were studied by enzymatic fingerprinting using a simultaneous enzyme treatment with endo-polygalacturonase (endo-PG) from *Kluyveromyces fragilis* and pectin lyase (PL) from *Aspergillus niger* to reveal the methyl-ester distribution patterns over the pectin backbone. Using HILIC-MS combined with HPAEC enabled the separation and identification of the diagnostic oligomers released. Structural information on the pectins was provided by using novel descriptive parameters such as degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme}) and degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}). This approach enabled us to clearly differentiate citrus pectins with various methyl-esterification patterns. The simultaneous use of PG and PL showed additional information, which is not revealed in digests using PG or PL alone. This approach can be valuable to differentiate pectins having the same DM and to get specific structural information on pectins and therefore to be able to better predict their physical and biochemical functionalities.

1. Introduction

Polysaccharides are the most abundant elements of the plant cell wall, determining the shape, size and many functional properties of the plant cell [1]. Pectin is a complex polysaccharide found in especially plant cell walls from fruits and vegetables [2] and has a key role in controlling the architecture of the primary plant cell wall and steering several plant processes as well as cell functions [1, 3, 4]. Traditionally, pectins are used in food products as a stabilizer, or a gelling and thickening agent. Dietary fibers, such as pectins, also play a significant role in the maintenance of health, both in gut fermentation and in immune modulation [5-8]

Pectins can be built up of four main structural elements, homogalacturonan (HG), rhamnogalacturonan I and II (RG I and RG II) and xylogalacturonan (XGA) [9]. Alfa-(1,4)-linked D-galacturonic acid (GalA) is the main building block of the HG which is the most prominent section of pectins, commonly present in amounts up to 60% of the total pectin structures [1]. The linear HG chain can be methyl-esterified at the carboxyl group at C-6 of GalA and, less commonly, also can be acetylated at the O-2 and/or O-3 position of the GalA residues [10].

Commercial pectin is mainly extracted from apple pomace and citrus peels [11] and since its structure strongly depends on the pectin source and extraction conditions, pectin structure might be highly diverse [12, 13]. Extracted pectins can be tailored further through targeted chemical- or enzymatic modifications to meet required functionalities [14]. Both the level and the distribution of the methyl-esters in the HG regions are key features within pectin's functionality [1, 3, 6, 15-17]. The percentage of methyl-esterified GalA residues within the HG backbone is defined as the degree of methyl-esterification (DM). Two main distribution patterns of methyl-esters have been described as random or blockwise [2, 18-20].

The methyl-esterification pattern of the pectin backbone was first quantitatively described by Daas et al. [21] as degree of blockiness (DB) which represents the amount of non-esterified mono-, di- and trigalacturonic acids released by enzymatic treatment of pectin using endo-polygalacturonase (endo-PG) from *Kluyveromyces fragilis,* relative to the total amount of non-esterified GalA residues present in the pectin [21]. To enable the action of endo-PG from *Kluyveromices fragilis* at least four consecutive non-esterified GalA residues are needed [21, 22]. Until now, DB and the related DB_{abs} (DB related to total amount of GalA residues present in the pectin) has been calculated from the amount of oligomers released as quantified in pectin digests by quite different methods like capillary electrophoresis (CE) and high performance anion exchange chromatography (HPAEC) analyses [19, 23-26]. Together, DB and DB_{abs} have been commonly used to differentiate methyl-esterification

patterns of pectins and are common parameters to characterize non-esterified blocks of GalA residues [19, 23, 27]. Details regarding the non-esterified block length and distribution of methyl-esters of pectins having a similar DM are rather difficult to define [28]. Pectins with similar DM and DB values can still show different patterns of methyl-esterification by having different sizes of non-esterified blocks [19]. To better understand pectin methyl-esterification patterns Ralet, Williams [27] described the degree of blockiness (DBMe) and absolute degree of blockiness ($DB_{abs}Me$) for the methyl-esterified regions in the homogalacturonan based on oligomers released upon pectin lyase (PL) digestion to study the highly methyl-esterified residues of pectins. Focusing either on the non-esterified pectin segments via the investigation of endo-PG digestion products or on the methyl-esterified sections released by the PL products explores only restricted sections of the entire pectin backbone [27]. Next to DB, DB_{abs}, DBMe and $DB_{abs}Me$, Remoroza et al. [29, 30] introduced new descriptive parameters, degree of hydrolysis by PG (DH_{PG}) and degree of hydrolysis by PL (DH_{PL}) for the enzymatic fingerprinting methyl-esterified and acetylated GalA sequences in sugar beet pectin. DH_{PG} and DH_{PL} are based on a combined enzymatic digestion by PL and endo-PG [29]. As yet, there has been no detailed investigation of the above-mentioned parameters, DH_{PG} and DH_{PL} for the analysis of non-acetylated pectins.

The main focus of the current research was to characterize and quantify the methyl-ester distribution of citrus pectins in more detail. Digestion using endo-PG acting preferably between unesterified GalA residues and PL requiring two neighboring methyl-esterified GalA residues was performed to describe methyl-ester distribution of 4 selected pectins. HPAEC-PAD/UV was used to identify and quantify GalA-oligomers released, although information on the level and location of methyl-esters are lost during analysis. HILIC-ESI-MS as complementing technique which preserves the methyl-esters present was used to distinguish methyl-esterified fragments, and to identify and quantify the diagnostic oligosaccharides released. The beauty of using this approach is that no pectin part remain high molecular weight and therefore un-analyzed. Novel parameters describing methyl-esterification are introduced and compared and different methyl-esterification patterns of pectins are discussed.

2. Materials and methods

2.1. Materials

Commercially extracted orange pectins O64 (DM 64%), O59 (DM 59%) and O32 (DM 32%) were provided by Andre Pectin (Andre Pectin Co. Ltd., Yantai, China). Commercially extracted lemon pectin L34 (DM 34%) was provided by CP Kelco (Copenhagen, Denmark). Endo-polygalacturonase (Endo-PG, EC 3.2.1.15; ID: 1027) from *Kluyveromyces fragilis* as described by [21]. A new batch of this enzyme was obtained from DSM (Delft, the Netherlands) and purified according to Pasculli, Geraeds [22]. In addition pectin lyase (PL, EC 4.2.2.10; ID: 1043) of *Aspergillus niger* [31, 32] was used to degrade the citrus pectins. Other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), VWR International (Radnor, PA, USA), or Merck (Darmstadt, Germany), unless stated otherwise.

2.2. Characterization of citrus pectins

Neutral sugar composition was analyzed after pretreatment with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by further acid hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). Neutral sugars released were derivatized and analyzed as their alditol acetates using gas chromatography [33], inositol was used as internal standard. Galacturonic acid content of the hydrolysate was determined by the automated colorimetric m-hydroxydiphenyl method [34, 35]. For the determination of the degree of methyl-esterification pectin samples were saponified using 0.1M NaOH for 24 h (1 h at 4 °C, followed by 23 h at room temperature). The methanol released was measured by a head-space gas chromatography (GC) method as previously described and consequently the DM was calculated [36].

2.3. Enzymatic hydrolysis

All citrus pectins were dissolved in 50 mM sodium acetate buffer pH 5.2 (5 mg/ml). The hydrolysis was performed at 40 °C by incubation of the pectin solution with PL for 6 hours followed by the addition of endo-PG and incubation for another 18 hours [30]. Enzyme doses were sufficient to degrade the entire pectin backbone into monomers within 6 hrs. Inactivation of enzymes was performed at 100 °C for 10 min and the digests were centrifuged (20,000 × g, 15 min, 20 °C). The supernatants obtained were analyzed by HPSEC, HPAEC-PAD/UV and UHPLC-HILIC-MS.

2.4. High performance size exclusion chromatography (HPSEC)

Pectin before and after enzymatic digestion were analysed by HPSEC on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of four TSK-Gel super AW columns) was used in series: guard column (6 mm ID × 40 mm) and columns 4000, 3000 and 2500 SuperAW (6 mm × 150 mm) (Tosoh Bioscience, Tokyo, Japan) at 55 °C. Samples (10 µl, 2.5 mg/ml) were eluted with filtered 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min. The elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). Pectin standards from 10 to 100 kDa were used to estimate the molecular weight distribution of the pectins [37].

2.5. High performance anion exchange chromatography (HPAEC)

The pectin digests were analyzed and subsequently quantified using an ICS5000 HPAEC-PAD (ICS5000 ED) (Dionex) equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.) and UV detection at 235 (Dionex). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C [38]. GalA DP 1–3 (Sigma–Aldrich, Steinheim, Germany) were used as standards for quantification. Oligomers above DP3 and unsaturated oligomers were quantified using the response from $GalA_3$ standard. Before the analysis pectin digests were diluted using ultra-pure water to 0.5 mg/ml. Samples (10 µl) were injected and eluted at a flow rate of 0.3 ml/min. The gradient profile was as follows: 0—55 min, 20—65% B; 55.1—60 min column washing with 100% B; finally, 60.1 to 75 min column re-equilibration with 20% B.

2.6. Ultra-High Pressure Liquid Chromatography HILIC-ESI-IT-MS

Pectin digests were analyzed using UHPLC in combination with electrospray ionization tandem mass spectrometry (ESI-IT-MS) on a Hydrophilic Interaction Liquid Chromatography (HILIC) BEH amide column (1.7 µm, 2.1 x 150 mm). Pectin digests were centrifuged (15,000 x g, 10 min, RT) and diluted (with 50% (v/v) aqueous acetonitrile containing 0.1% formic acid to a final concentration of 1 mg/ml). The eluents used were (A) 99:1% (v/v) water/acetonitrile (water/ACN); (B) 100% ACN, both containing 0.1% formic acid with a flow rate of 400 µl/min. The following elution profile was used: 0—1 min, isocratic 80% B; 1—46 min, linear from 80% to 50% B; followed by column washing: 46—51 min, linear from 50% to 40% B and column re-equilibration; 52—60 min isocratic 80% B. The oven temperature was set at 40°C. The injection volume was 1 µl. Mass spectra were acquired over the scan range m/z 300—2000 in

the negative mode. A heated ESI-IT ionized the separated oligomers in an LTQ Velos Pro Mass Spectrometer (UHPLC-ESI-IT-MS) coupled to the UHPLC.

2.7. Descriptive pectin parameters

2.7.1. Determination of degree of blockiness and absolute degree of blockiness

The degree of blockiness (DB) is calculated as the number of moles of GalA residues present as non-esterified mono-, di- and triGalA released by endo-polygalacturonase related to the total amount of non-esterified GalA residues present and expressed as a percentage (Equation 1) [19, 21, 23]. The absolute degree of blockiness (DB_{abs}) is calculated as the amount of non-esterified mono-, di- and triGalA residues released by endo-PG expressed as the percentage of the total GalA residues present in the pectin (Equation 2) [19, 23]. The amount of GalA monomer, dimer, trimer released from the digested pectins was determined by HPAEC-PAD and corrected for partially methyl-esterified triGalA levels using HILIC-ESI-IT-MS data. GalA and $GalA_2$ and $GalA_3$ (Sigma-Aldrich, Steinheim, Germany) were used for quantification. DB and DB_{abs} were calculated using the following formulas:

$$DB = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_n\text{released}]_{\text{non-esterified}} \times n}{[\text{total non-esterified GalA in the polymer}]} \times 100 \quad (1)$$

$$DB_{abs} = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_n\text{released}]_{\text{non-esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (2)$$

2.7.2. Determination of degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme})

To get a clear picture of the partially methyl-esterified HG region of citrus pectins, a new parameter DB_{PGme} was used. Using the amounts of individual saturated and methyl-esterified oligosaccharides present after digestion by endo-PG, the formula of degree of hydrolysis by PG was modified [30] in order to distinguish between completely non-esterified blocks and partially methyl-esterified regions released by PG. As equation 3 shows, DB_{PGme} is calculated as the number of moles of galacturonic acid residues present in the digest as saturated, methyl-esterified GalA oligomers DP 3–8 per 100 moles of the total GalA residues in the pectin polymer (saturated DP 2 is never methyl esterified).

$$\mathbf{DB_{PGme}} = \frac{\sum_{n=3\text{-}8} [\text{saturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (3)$$

2.7.3. Determination of degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme})

Beside the saturated partially esterified residues as degraded by PG, the number of unsaturated oligomers by the simultaneous PL action is determined as well. The DB_{PLme} quantifies the amount of unsaturated and methyl-esterified GalA oligomers (DP 2–8) released by PL. DB_{PLme} is based on the previous concept $DB_{abs}Me$ for highly methyl-esterified stretches [27]. $DB_{abs}Me$ is defined as mole of GalA residues present as unsaturated methyl-esterified GalA oligomers per 100 mole of total GalA units in the polymer as released after PL digestion [27]. In our study a similar approach of Ralet et al. was used, but in this case PG and PL were used simultaneously instead of PL alone [27] resulting in slightly different PL-derived oligomers. As shown by equation 4, all GalA residues present as unsaturated partly methyl-esterified oligomers (DP 2–8), released by PL action were quantified and expressed as degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}).

$$\mathbf{DB_{PLme}} = \frac{\sum_{n=2\text{-}8} [\text{unsaturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (4)$$

3. Results and discussion

3.1. Characteristics and parameters of pectin samples used in this study

Pectins used in this study were characterized for GalA content, neutral sugar composition, molecular weight distribution and degree of methyl-esterification. The characteristics of the pectins are given in table 1.

Table 1. Characteristics of citrus pectin samples used in this study.

Pectin	Rha	Ara	Gal	Glc	GalA[b]	Total	Mw (kDa)[d]	DM (%)[e]
			mol %			(w/w%)[c]		
O64[a]	0 ± 0.30	7 ± 0.41	7 ± 0.29	1 ± 0.01	82 ± 1.2	86 ± 2.8	92	64 ± 2.6
O59	1 ± 0.04	3 ± 0.22	9 ± 0.03	3 ± 0.04	84 ± 0.62	83 ± 4.3	87	59 ± 2.1
O32	1 ± 0.04	3 ± 0.05	6 ± 0.09	1 ± 0.02	89 ± 0.02	87 ± 2.9	77	32 ± 1.9
L34	1 ± 0.16	3 ± 0.09	6 ± 0.25	1 ± 0.21	89 ± 0.73	65 ± 2.9	107	34 ± 3.1

[a] O: orange origin, L: lemon origin, Number: DM. O64 = Orange pectin with a DM of 64
[b] Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, GalA = Galacturonic Acid.
[c] Total sugar content in w/w%
[d] Molecular weight (Mw) as measured by HPSEC.
[e] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample.

Two pairs of pectins were selected because each pair have similar DM and similar features. The chemical characteristics of pectins are typical for homogalacturonan type pectins from citrus origin [1, 39] and only small variations in the neutral sugar content, GalA content and the DM of HM and LM pectins are present as can be seen in Table 1. The molecular weight distribution of all four pectins are rather similar with a Mw around 90 kDa (see also figure 1), which is in accordance with previous studies [19, 40].

3.2. Enzymatic fingerprinting of citrus pectins

Enzymatic fingerprinting of pectins using one single enzyme activity is a well-known approach for structural characterization since enzymes have established substrate specificities. In this study however, in order to study the methyl-ester distribution in commercial citrus pectins, pectins O64, O59, O32 and L34 were degraded using a combination of two pure and well defined pectin enzymes: endo-PG and PL. Pectin degradation was followed by HPSEC

with RI detection. The enzyme-treated citrus pectins showed a shift to low molecular weight oligomers (< 2.5 kDa) containing information on methyl-esterification as will be discussed in section 3.3.

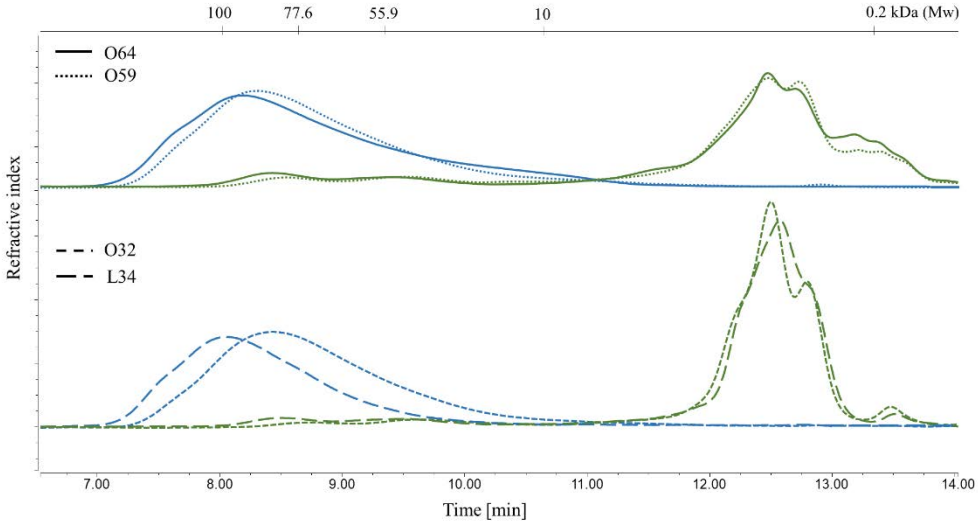

Figure 1. HPSEC elution patterns of O64, O59, O32 and L34 pectins before (●blue line) and after (●green line) digestion by homogalacturonan degrading enzymes: PL and endo-PG. Molecular weights of pectin standards (in kDa) are indicated.

After degradation, the diagnostic oligomers formed show similar low Mw (RT 11-14 min) for both pairs of similar DM pectins, however it can be already seen from the peak shape that the degradation products might differ. What stands out in the chromatogram is that endo-PG combined with PL degraded the citrus pectins almost completely into oligomers of Mw < 2.5 kDa. This complete enzymatic degradation of the pectin by a combination of enzymes to oligosaccharides is a considerable improvement compared to the use of single enzymes like endo-PG, exo-PG or PL, all having their own DM-dependency, to convert pectins only partly into diagnostic oligomers [19, 21, 27, 41].

3.3. Characterization and quantification of the diagnostic oligomers

The differences between the methyl-ester distribution patterns of pectins has till now mainly been described by the parameters DM, DB, and DBMe and in addition DH_{PG}, DH_{PL} are used to describe acetylated pectins [19, 21, 27, 30]. HPAEC-PAD/UV of the endo-PG and PL degradation products of citrus pectins allowed the separation, identification and quantification of monoGalA and both saturated and unsaturated GalA oligomers ranging from degree of polymerization (DP) 2-7 (figure 2). However, as a consequence of the high pH

(pH 12) used during the HPAEC separation, information on the methyl-esterification of the different oligomers is lost.
In the HPAEC saturated oligomers eluted earlier, while unsaturated oligomers eluted later, and in most cases they were nicely separated, however uDP1 (unsaturated GalA DP1) is not present and DP5 and uDP3 are coeluting. Fortunately they can be distinguished with the help of the UV signal. Figure 2 reveals that the same type of oligomers were released after PG and PL treatment of the citrus pectins, but in quite different quantities for the various pectins. Especially, the similar-DM pectins O32 and L34 show rather different patterns, while patterns are rather similar for O64 and O59. Quantification of the oligosaccharides showed that the amount of saturated DP1-3 produced after degradation was higher in the O64 and L34 than in the similar DM, O59 and O32 pectins which means that O64 and L34 have more non-esterified GalA blocks present being accessible to PG.

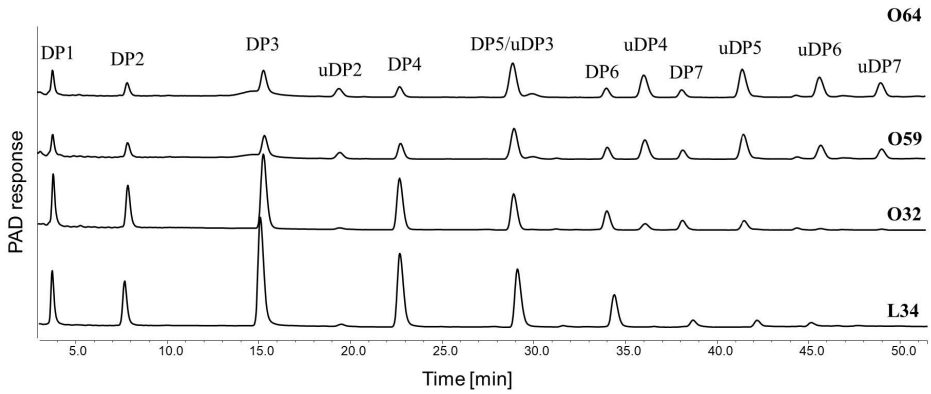

Figure 2. HPAEC-PAD elution patterns of endo-PG and PL digests of O64, O59, O32 and L34 pectins after 24h incubation detected by PAD. Peak annotation: DP4, saturated DP4 GalA oligosaccharide; uDP4, unsaturated DP4 GalA oligosaccharide.

What can be clearly seen in figure 2 is the difference between the low DM pectins regarding the unsaturated oligomers released. In the O32 digest, there were higher amounts of unsaturated products present such as uDP2, uDP4 and uDP5 compared to L34. Despite the presence of dominantly non-esterified GalA residues in PG-degradable sequences, the methyl-esters still are positioned differently over the backbone of these two LM pectins causing the PL to act and to act differently. Despite rather similar oligosaccharide structures released for O59 and O64, still small differences can be observed in the amounts released. These results already confirm the presence of different methyl-esterification patterns over the pectin backbone in pectins having similar DM. Which means that, pectins with similar DM can have different patterns of methyl-esterification.

3.4. Structure elucidation of the generated oligosaccharides after enzymatic digestion

To tackle the limitations of HPAEC due to the removal of methyl-esters at high pH (pH 12) [42], HILIC-MS was employed to separate and identify methyl-esterified oligomers [43]. Peak annotation has been done based on the m/z of the GalA oligosaccharides, and relative abundance of selected DPs has been obtained after integration of peak areas in the ion chromatograms (figure A.1. showing DP3 as an example). Since saturated dimer is only present as non-esterified oligomer, and the saturated DP4 only as methyl-esterified oligomer, saturated DP3 had to be checked for methyl-esters. Following the quantification of DP 1-7 and uDP 2-7 using the HPAEC-PAD, the relative abundance of oligomers obtained from HILIC-MS was used to differentiate between differently methyl-esterified and non-esterified oligomers within one DP.

Figure 3 illustrates the HILIC elution patterns of the enzyme digests of the four citrus pectins. It is shown that the main degradation products are present in all digests but at different ratios, demonstrating different methyl-ester distribution in the same DM pectins. Besides the unsubstituted dimer (2^0) and trimer (3^0), partially methyl-esterified saturated and unsaturated GalA oligomers of different DPs are present as main degradation products as illustrated by mono-esterified trimer, mono- and di-esterified tetramer, mono-, di- and tri-esterified pentamer and so on. All these oligomers with different levels of methyl-esterification can be easily separated and not only the saturated, but also the unsaturated galacturonic acid oligomers. The sequence of elution of GalA oligomers is based on clustering oligomers of the same charge although larger oligomers eluted slightly later than smaller oligomers having the same net charge, due to small differences in charge density [44]. For example: retention times of DP $4^1 < 5^2 < 6^3 < 7^4$ increase with the number of GalA residues present in the oligomer while they all have the same net charge.

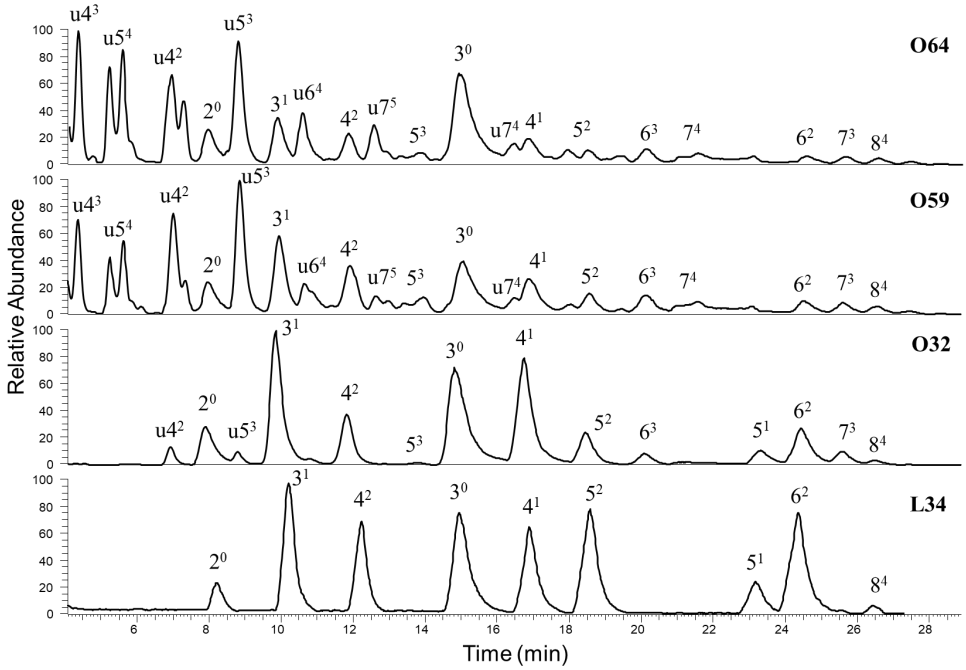

Figure 3. HILIC-MS base peak elution pattern of O64, O59, O32 and L34 digested by homogalacturonan degrading enzymes endo-PG and PL. Peak annotation: 3^1, saturated DP3 GalA oligosaccharide having one methyl-ester; $u5^3$, unsaturated DP5 GalA oligosaccharide having three methyl-esters.

The HILIC chromatogram of O64 digest is different from O59 digest since showing different relative intensities for the various oligosaccharides. The amounts of unsaturated highly methyl-esterified oligomers such as $uDP5^4$, $uDP6^4$ and $uDP7^5$ in the O64 digest were higher than in the O59 digest, suggesting more densely methyl-esterified regions in O64 compared to O59. The small saturated non-esterified oligosaccharides DP 2^0 and 3^0 are slightly higher in O64 compared to O59, pointing to a more blockwise distribution non-methyl esterified GalA residues within O64. In addition, the levels of unsaturated low methyl-esterified oligomers such as $uDP3^1$, $uDP4^2$ and saturated $DP4^1$, $DP5^2$ were higher in O59 pectin pointing to the presence of more randomly distributed methyl-esterified GalA residues in O59 compared to O64. The ratios of different oligomers differ highly in the digests of the two similar-DM pectins. For example, the ratio of $uDP4^2$: $uDP5^3$: $uDP6^4$: $uDP7^5$ in O64 and O59 are rather different 17:24:38:22 for O64 and 34:51:50:42 for O59. While DM64 has higher amounts of $uDP6^4$ and $uDP7^5$, in DM59 $uDP4^2$ and $uDP5^3$ are higher.

For the low DM pectins, in the L34 digest hardly any unsaturated products like uDP4[2] and uDP5[3] were detected which is expected as PL has low activity on low DM pectins, however in the O32 digest those unsaturated products were found. This result may be explained by the fact that the number and distribution of methyl-esters affects the activity of PL. The enzyme can cleave partially methyl-esterified GalA residues, but its activity towards pectins having DM<50 is rather limited [45, 46]. Surprisingly, O32 must have some PL degradable residues where methyl-esters are more clustered on the backbone. In L34 pectin digest the quantity of saturated methyl-esterified oligomers released and also the ratio of e.g., saturated DP 4^2: 5^2: 6^2 is higher than in O32. This suggests a specific pattern of methyl-esterification, having stretches of non-esterified GalA residues interrupted with a few methyl-esterified GalA residues. Altogether, absolute as well as relative amounts of the various oligomers clearly differ for different pectins, even when having the same DM and may add to a detailed characterization of pectin's methyl-esterification patterns. Taking the relative abundance of individual oligosaccharides which are identified obtained from HILIC and applying those ratios on the easily separated and quantified oligomers from HPAEC can be beneficial to explain the differences in pectin structure and help to explain pectin functionality.

3.5. Investigation of pectin methyl-esterification patterns

Previously the methyl-esterified segments present in pectins were described by DBMe and $DB_{abs}Me$ based on PL digestion alone and for the non-esterified segments DB and DB_{abs} were used based on oligomers released by PG alone [19, 21, 27]. However, it seems that the precise methyl-ester distribution patterns are not yet clearly revealed, described and understood by these parameters. By the simultaneous PL and PG digestion and by the combination of HPAEC and HILIC high throughput analysis is possible as all the pectin oligomers can be examined in a very short time. By calculating the DB_{PGme} and DB_{PLme} based on simultaneous degradation by PG and PL, additional information can be revealed on the methyl-esterification patterns of the citrus pectins and pectins can more readily be compared based on these parameters. DB_{abs} quantifies unsubstituted mono-, di- and tri GalA oligomers as released by PG related to all GalA present in the pectin, D_{BPGme} does quantify PG released saturated and partly methyl-esterified random segments of the pectin and DB_{PLme} quantifies PL released unsaturated and highly methyl-esterified oligomers released from the pectin, therefore by these three parameters the entire pectin backbone can be described.

Table 2. Descriptive parameters of citrus pectins used in this study.

Pectin (DM)	DB (%)[b]	DB_{abs} (%)[c]	DB_{PGme} (%)[d]	DB_{PLme} (%)[e]
O64[a]	37	13	18	65
O59	28	11	30	53
O32	41	27	67	11
L34	50	33	95	5

[a] O: orange pectin, L: lemon pectin. Number: DM. O64 = Orange pectin with a DM of 64
[b] Degree of blockiness (DB): the amount of non-esterified mono-, di- and triGalA per 100 mol of the non-esterified GalA in the sample.
[c] Absolute degree of blockiness (DB): the amount of non-esterified mono-, di- and triGalA per 100 mol of total GalA in the sample.
[d] Degree of blockiness by endo-PG (DB_{PGme}): the amount of saturated methyl-esterified galacturonic residues per 100 mol of total galacturonic acid in the sample.
[e] Degree of blockiness by PL (DB_{PLme}): the amount of methyl-esterified unsaturated galacturonic oligomers per 100 mol of total galacturonic acid in the sample.

Table 2 shows these descriptive parameters for the four pectins used in this study. It can be seen that, even though the DM of both low DM and high DM pectins are rather similar, especially the DB_{PGme} and DB_{PLme} parameters differ from each other. The DB_{PGme} is 40% lower while the DB_{PLme} is 23% higher in O64 than in pectin O59. O64 thus has, next to non-esterified blocks, also blocks with methyl-esterified residues. In contrast to first thoughts that an equal DB_{abs} of two pectins would indicate similar pectin methyl-ester distributions, the different DB_{PLme} and DB_{PGme} of O64 and O59 suggest much more refined structural differences. In O59 there are more PG degradable methyl-esterified GalA residues, which indicates more randomly methyl-esterification next to having highly methyl-esterified residues degradable also by PL. DB_{PLme} and DB_{PGme} complement the previous research describing pectins using DB [19, 23] while also adding an extra dimension by revealing differences in the methyl-esterified regions of pectins using both PL and PG simultaneously. Figure 4 visualizes the differences in methyl ester patterns from the two high DM pectins. The oligomers released by PG and PL in the digests are highlighted as also included in the formulas and the hypothetical representation of the parental molecule is visualized.

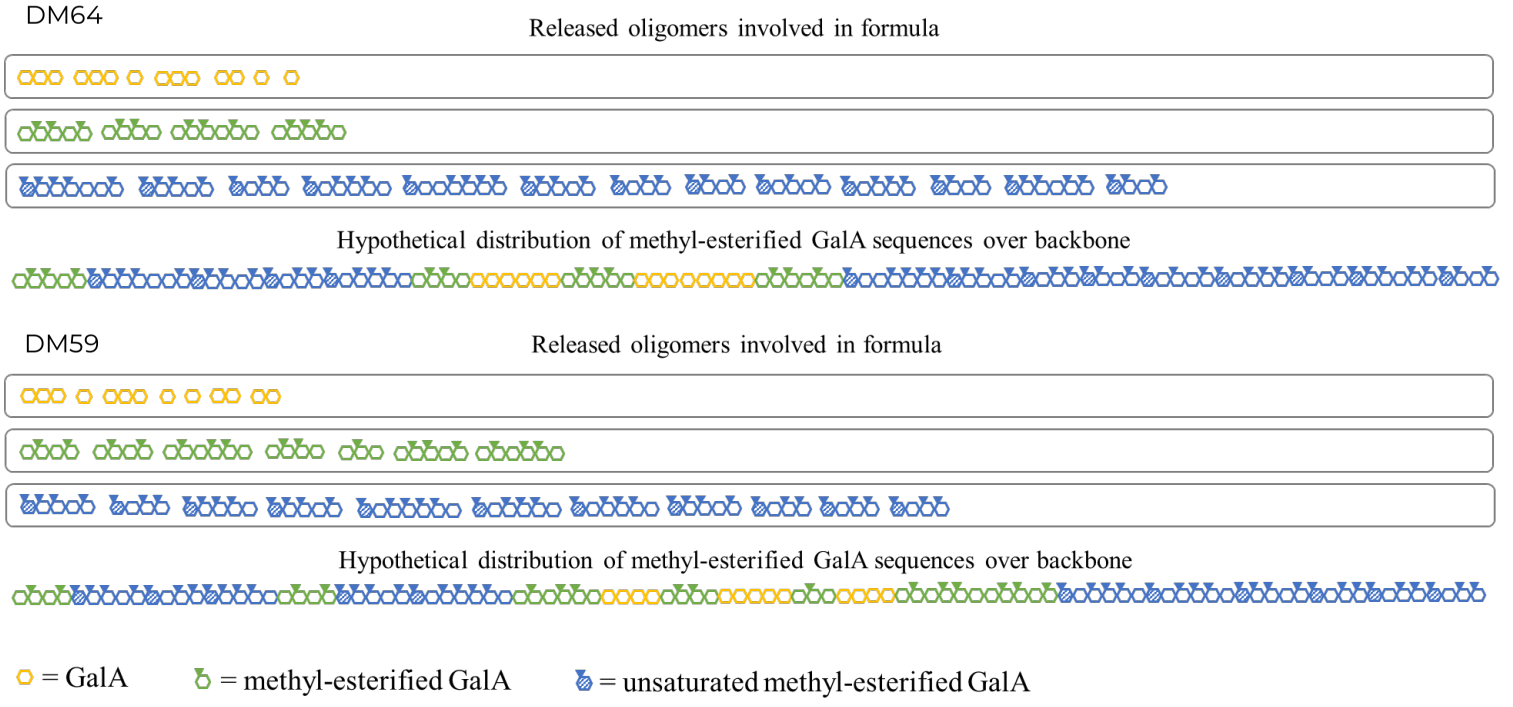

Figure 4. Schematic representation of enzymatic digestion with endo-PG of *Kluyveromyces fragilis* and PL on two ~60% DM pectins, O64 and O59 having different methyl-ester distributions. The released diagnostic oligomers can be analysed and quantified on HPAEC and HILIC and consequently the descriptive parameters, such as DB, DB_{abs}, DB_{PGme} and DB_{PLme} can be calculated. The precise location of the released oligomers could not be determined.

The relative abundance of the different oligomers as released by the combination of endo-PG and PL differs to a large extent in the pectins studied. As expected O64 served as good substrate for PL. Interestingly, also oligomers such as $DP7^5$ and $uDP7^5$ were present in the digests, which in theory could have been degraded further by PL, but this may be explained by the pattern of methyl-ester distribution within the oligomer, not matching with the specificity of the enzyme [42, 45]. Larger differences were found between pectin digests having highly methyl-esterified oligomers. Good examples for the densely methyl-esterified segments are the unsaturated $uDP4^3$, $uDP5^4$ and $uDP6^5$ oligosaccharides released by PL from O64 pectin in 30-50% higher amounts than from O59 pectin. O59 pectin has less methyl-esterified GalA stretches, degradable by PL, in addition to non-esterified stretches, degradable by PG, releasing both more non-esterified GalA DP1-3 and more methyl-esterified GalA sequences which could not be released/degraded by PL (figure 4). The presence and length of the methyl-esterified oligomers released by PG represent the pattern of methyl-esterification outside any block and are not covered by DM nor DB, but are now covered by DB_{PGme} and DB_{PLme}.

For low DM pectins it was found, as hypothesized, that they are favorable substrates for endo-PG and mainly saturated oligomers were released. However, more unexpected, differences can still be found in methyl-ester distribution patterns. Interestingly in case of O32, the level of methyl-esterified products released by PL, the DB_{PLme}, is more than doubled compared to L34 having a similar DM, at the expense of partly methyl-esterified GalA oligomers being released by PG. Together, this suggests a less random pattern of methyl-esterification for L34. Furthermore, in O32 mainly less methyl-esterified oligomers are present like $DP4^1$, $DP5^1$ or $DP6^3$ which relates to a more random pattern of methyl-esters in lower DB pectin.

4. Conclusion

The main goal of the current study was to elucidate pectin methyl-esterification patterns by using combined endo-PG and PL digestion on two pairs of commercial citrus pectins representing either high or low DM pectins. When using HPAEC alone, the saturated and unsaturated GalA oligomers can be easily separated and quantified. In addition, with HILIC the different methyl-esterified oligomers in pectin digests having the same DP can be easily differentiated. Information on both the saturated (non)methyl-esterified oligo galacturonides released by PG and the methyl-esterified unsaturated oligo galacturonides released by PL, can now be used to simply characterize pectins with various structural parameters faster and in more detail. It was demonstrated that pectin methyl-esterification patterns differ highly, even in pectins having similar DM and DB. The efficient separation and identification of oligomers using HILIC demonstrate the value of the analysis of citrus pectin digests and can provide understanding between pectin fine structure and functionality. Combing endo-PG and PL digestion of pectin and consequently quantifying the entire homogalacturonan region, provided more details on the methyl-esterification patterns in citrus pectins, beyond the degree of blockiness. It is possible now to characterize methyl-esterified pectins on a higher level by recognizing patterns between fully non-esterified and fully esterified segments. This approach can be useful to differentiate between pectins having the same levels of methyl-esterification but different physical and biochemical functionalities and to explain these differences in applications.

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Appendix

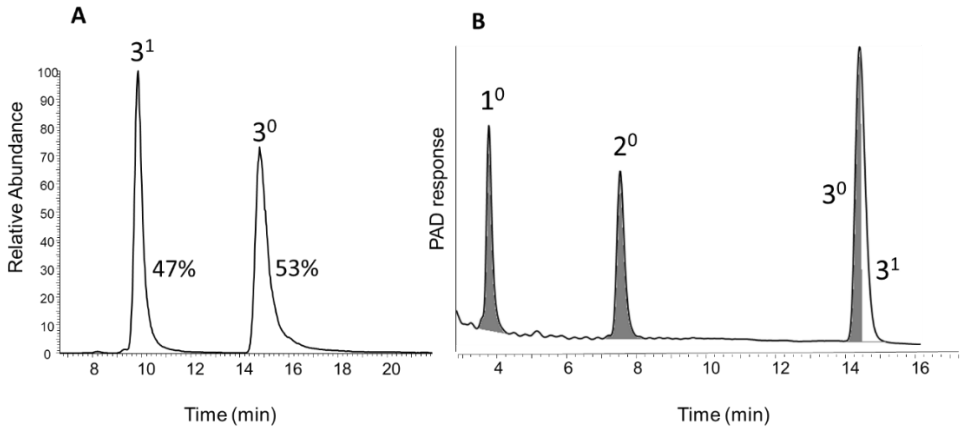

Figure A.1 A. UPLC-HILIC-MS profile of O32 digested by endo-PG and PL enzymes with the selection of saturated $GalA_3$ masses. Peak annotation: 3^1: saturated DP 3 having one methyl-ester. Showing the relative abundance of GalA DP 3^0 and 3^1.
B. HPAEC-PAD elution pattern of DP1-3 from the same O32 pectin after PG and PL digestion indicating that the $GalA_3$ area covers DP 3^1 and 3^0 in different proportions.

Chapter 3

The impact of the level and distribution of methyl-esters of pectins on TLR2/1 dependent anti-inflammatory responses

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Abstract

Pectins have anti-inflammatory effects via Toll-like receptor (TLR) inhibition in a degree of methyl-esterification-(DM)-dependent manner. However, pectins also vary in distribution of methyl-esters over the galacturonic-acid (GalA) backbone (Degree of Blockiness - DB) and impact of this on anti-inflammatory capacity is unknown. Pectins mainly inhibit TLR2/1 but magnitude depends on both DM and DB. Low DM pectins (DM18/19) with both low (DB86) and high DB (DB94) strongly inhibit TLR2/1. However, pectins with intermediate DM (DM43/DM49) and high DB (DB60), but not with low DB (DB33), inhibit TLR2/1 as strongly as low DM. High DM pectins (DM84/88) with DB71 and DB91 do not inhibit TLR2/1 strongly. Pectin-binding to TLR2 was confirmed by capture-ELISA. In human macrophages, low DM and intermediate DM pectins with high DB inhibited TLR2 1 induced IL-6 secretion. Both high number and blockwise distribution of non-esterified GalA in pectins are responsible for the anti-inflammatory effects via inhibition of TLR2/1.

1. Introduction

A lower intake of dietary fibers in Western society compared to more traditional diets is associated with a higher chance of developing diseases with a dysregulated immunity such as type 2 diabetes, obesity, inflammatory bowel disease, and autoimmune disorders [1–5]. In contrast, a high dietary fiber intake in traditional societies coincided with a lower frequency of those diseases [1,6]. The mechanisms by which dietary fiber intake prevents immunity-related disease are not fully understood. Several studies have shown that dietary fibers can influence immunity by supporting intestinal microbiota and enhancing the production of metabolic fermentation products such as short-chain fatty acids (SCFA), and aryl hydrocarbon receptor (Ahr)-ligands or other microbial-derived molecules [7,8]. Moreover, dietary fibers are also known to directly stimulate the immune system [9,10] by binding to Toll-like receptors (TLRs) [11–13]. TLRs are a family of pattern recognition receptors (PRRs) that play an important role in intestinal immune regulation [14]. PRRs serve as sensors for innate immunity and may after activation stimulate transcription factors NF-κB and AP-1, which induce upregulation of pro- and anti-inflammatory genes, depending on the activated receptor interactions [15]. This may activate not only innate immune responses but also activate adaptive immune responses [16–18]. In the intestine, TLRs are expressed on most immune and gut epithelial cells [19,20]. Each TLR recognizes specific pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or food-associated molecules [15].

Pectin is one of the dietary fiber molecules with TLR binding capacity and has been shown to have anti-inflammatory effects depending on its chemical structure [12,13,21–24]. Native plant pectin consists of homogalacturonan, rhamnogalacturonan I (RG-I), and II (RG-II). RG-I segments consist of a backbone of repeating disaccharide backbone structures of alternating GalA and rhamnose residues. The rhamnose residues can be branched with neutral side chains. RG-II segments contain a backbone of GalA residues, with short side chains which contain 12 different sugar residues [25,26]. These pectins consist mainly (≥ 70%) of linear 1,4-D-galacturonic acid (GalA) (homogalacturonan) segments and minor amounts of branched rhamnogalacturonan segments [27]. The homogalacturonan backbone can be methyl-esterified (Figure 1), and the amount of esters on the backbone is referred to as the degree of methyl-esterification (DM) [28]. Dependent on the DM, pectins have different functional properties. Sahasrabudhe et al., 2018 showed that TLR2/1 was inhibited in a DM-dependent manner by lemon

pectins in which a gradual decreasing DM increased TLR2/1 inhibiting properties of pectins. In addition, TLR2 ectodomains bound stronger to pectins with lower DM pectins than to pectins with a higher DM [13]. However, pectins not only differ in DM but also in the distribution of methyl-esters over the backbone. The degree of blockiness (DB) is a structural parameter for the distribution of non-esterified GalA residues in pectins (Figure 1). When comparing pectins with similar DM, high DB (HB) pectins have a more blockwise distribution of non-esterified GalA residues compared to low DB (LB) pectins. This is in contrast to LB pectins which have a more random distribution of non-esterified GalA residues (Figure 1) [29]. When comparing pectins with a different DM (DM40 and DM80), but a similar DB, the total number of non-esterified GalA residues that are blockwise distributed is larger on pectins with DM40 than on pectins with DM80. This is because the DM40 pectin contains a larger number of non-esterified GalA residues than DM80 pectin (Figure 1) [29]. How DB contributes to TLR signaling is not known.

In the present study, it was hypothesized that the level and distribution of methyl-esters in pectin determine the efficacy of pectins as TLR signaling molecules. Therefore, the relationship between pectin structures and Toll-like receptor signaling was determined by comparing the impact of pectins with different DM and DB on activation or inhibition of different TLRs in reporter cells expressing TLRs. First, structurally different orange and lemon pectins were studied on having similar DM-dependent effects on activation and inhibition of TLR2, 2/1, 2-6, 3, 4, 5, 7, 8, and 9. Next, it was studied which combination of the structural parameters DM or DB induced the most pronounced TLR2/1 inhibition. Furthermore, the effects of pectins on TLR2 binding were also studied. In addition to the effects of pectins on TLR2 reporter cell line, the stimulating or attenuating effects of pectins on cytokine secretion by human macrophages in vitro were studied.

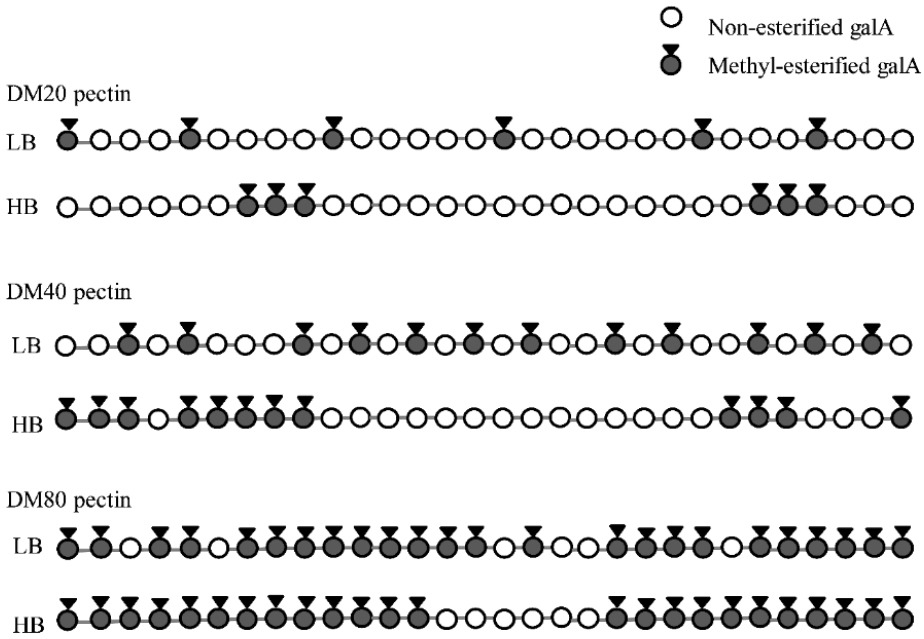

Figure 1. *Schematic representation of low DB and high DB structures of pectins with DM20, DM40 and DM80. Homogalacturonan pectins consist of a galacturonic acid (GalA) backbone structure in which GalA residues can be methyl-esterified (degree of methyl-esterification; DM). Low degree of blockiness (LB) pectins contain a more random distribution of non-esterified GalA residues, whereas high degree of blockiness (HB) pectins contain a more blockwise distribution of non-esterified GalA residues [29].*

2. Materials and methods

2.1. Pectin samples

Commercially extracted pectins of orange origin (DM32, DM64) were obtained from Andre Pectin (Andre Pectin Co. Ltd., Yantai, China). Pectins of lemon origin DM18, DM19, DM33, DM43, DM49, DM52, DM84 and DM86) were obtained from CP Kelco (Lille Skensved, Denmark).

2.2. Cell lines

To study the influence of pectins on Toll-like receptor (TLR) signaling various HEKBlueTM reporter cell lines (Invivogen, Toulouse, France) were used [12,30]. These reporter cell lines express Soluble Embryonic Alkaline Phosphatase (SEAP). The SEAP reporter gene is placed under the control of an NF-κB and an AP-1 responsive promotor. Upon activation of the TLRs by a specific agonist, high levels of intracellular NF-κB will lead to the secretion of SEAP which can be quantified by QUANTI-Blue (Invivogen, Toulouse, France). HEK-Blue cells containing a construct of human TLR2, 3, 4, 5, 7, 8, or 9 (Invivogen) were used to study the effect of pectins on single TLRs. HEK 293/hTLR2- HA (Invivogen) was used for studying the interaction of TLR2 and pectins. All HEKBlueTM and 293/hTLR2-hemagglutinin (HA) cells were cultured in DMEM culture media (Lonza, Basel Switzerland) containing 10% de-complemented Fetal Calf Serum, 50U/ml Penicillin (Sigma, St. Louis, MO, USA), 50 µg/ml Streptomycin (Sigma), 100 µg/ml Normocin (Invivogen) according to the manufacturer's instructions. The reporter cells were cultured for three passages before they were maintained in a selection medium (Table 1). Human monocytic THP-1 cells (ATCC, Manassas, USA) were cultured in RPMI 1640 medium (Lonza, Bornem, Belgium) with 10% fetal bovine serum (Sigma-Aldrich, MO USA), 2 mM L-glutamine (Lonza, Belgium), 1 mM sodium pyruvate (Lonza, Belgium), 0.05 mM 2-mercaptoethanol (Scharlau, Barcelona, Spain), 60 µg/ml gentamicin sulfate (Lonza, Belgium), 2.2 µg/ml amphotericin B solubilized (Sigma).

Table 1. *TLR reporter cell lines and selection of antibiotics*

Cell line (Invivogen)	Selection antibiotics (Invivogen)
HEK-Blue hTLR2	HEK-Blue 1X
HEK-Blue hTLR3	Zeocin (100 µg/ml) Blasticidin (30 µg/ml)
HEK-Blue hTLR4	HEK-Blue 1X
HEK-Blue hTLR5	Zeocin (100 µg/ml) Blasticidin (30 µg/ml)
HEK-Blue hTLR7	Zeocin (100 µg/ml) Blasticidin (10 µg/ml)
HEK-Blue hTLR8	Zeocin (100 µg/ml) Blasticidin (30 µg/ml)
HEK-Blue hTLR9	Zeocin (100 µg/ml) Blasticidin (10 µg/ml)
HEK293T TLR2-HA	Blasticidin (50 µg/ml)

2.3. Determination of monosaccharide composition

The neutral sugar composition of the pectins was analyzed after pre-hydrolysis with 72% (w/w) H2SO4 (1 h, 30 °C) followed by further hydrolysis with 1 M H2SO4 (3 h, 100 °C) [31]. Neutral sugars released were reduced with sodium borohydride to form their corresponding alditols and then acetylated to yield their volatile derivatives. These alditol acetates were separated and quantified by gas-liquid chromatography (GLC Trace 1300; Interscience Focus-GC, Thermo Fisher Scientific) as described by Englyst and Cummings [31] equipped with a flame-ionization detector (FID) and a 15 meter DB225 column (Agilent J&W, Santa Clara, CA, USA). Inositol was used as an internal standard. The uronic acid content was determined by the automated colorimetric m-hydroxydiphenyl method [32].

2.4. Enzymatic hydrolysis

The enzymes used in this study were pectin lyase (EC 4.2.2.10) [33] and endo-polygalacturonase from *Kluyveromyces fragilis* [29]. All citrus pectins were dissolved in 50 mM sodium acetate buffer pH 5.2 (5 mg/ml). The hydrolysis was performed at 40 °C by incubation of the pectin solution with PL for 6

hours followed by the addition of endo-PG and incubated for another 18 hours. Enzyme doses were sufficient to degrade the pectin backbone within 24 hrs. Inactivation of enzymes was performed at 100 °C for 10 min and the digests were centrifuged (20,000 × g, 20 °C, 15 min). The supernatants obtained were analyzed by high-performance size exclusion chromatography (HPSEC), high-performance anion exchange chromatography (HPAEC-PAD/UV) and Ultra-High Pressure Liquid Chromatography HILIC-ESI-IT-MS^n.

2.5. High-performance size exclusion chromatography (HPSEC)

Pectin before and after enzymatic digestion were analyzed using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of four TSK-Gel super AW columns (Tosoh Bioscience, Tokyo, Japan) was used in series: guard column (6 mm ID × 40 mm) and the columns TSK super AW 4000, 3000 and 2500 (6 mm × 150 mm). The column temperature was set to 55 °C. Samples (10 µL, 2.5 mg/ml) were eluted with filtered 0.2 M NaNO3 at a flow rate of 0.6 ml/min. The elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). The HPSEC system was calibrated using polydisperse pectin standards having molecular weights ranging from 10 to 100 kDa as estimated by viscosimetry [34]. To display clearly the molecular weight of pectins larger than 100 kDa, 150 kDa has been calculated from the standards. Molecular weights presented were estimated at the top of the curve, despite slight differences in elution patterns of the various pectins pointing to differences in polydispersity.

2.6. High-performance anion exchange chromatography (HPAEC)

The pectin digests were analyzed and subsequently quantified using an ICS5000 High-Performance Anion Exchange Chromatography system with Pulsed Amperometric detection (ICS5000 ED) (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C [35]. GalA DP 1–3 (Sigma– Aldrich, Steinheim, Germany) were used as standards for quantification. UV monitoring of the eluent at 235nm allowed the identification of unsaturated oligoGalAs as released by the action of pectin lyase through the presence of a double bond. Before the analysis pectin digests were diluted using ultra-pure water to 0.5 mg/ml. They were injected (10 µl) and eluted at a flow rate of 0.3 ml/min. The gradient profile for elution was as follows: 0—55 min, linear 20—

65% B; 55.1—60 min column washing with 100% B; finally, column re-equilibration with 20% B from 60.1 to 75 min.

2.7. Ultra-High Pressure Liquid Chromatography HILIC-ESI-IT-MS

Pectin digests were analyzed using UHPLC in combination with ESI-IT-MS on a Hydrophilic Interaction Liquid Chromatography (HILIC) BEH amide column 1.7 µm, 2.1 x 150 mm (Thermo Scientific). Pectin digests were centrifuged (15000xg, 10 min, RT) and diluted (with 50% (v/v) acetonitrile containing 0.1% formic acid, to a final concentration of 1 mg/mL). The eluents used were (A) 99:1% (v/v) water/acetonitrile (water/ACN); (B) 100% ACN, both containing 0.1% formic acid with a flow rate of 400 µL/min. The following elution profile was used: 0—1 min, isocratic 80% B; 1—46 min, linear from 80% to 50% B; followed by column washing: 46—51 min, linear from 50% to 40% B and column re-equilibration; 51.1—60 min isocratic 80% B with a flow rate of 400 µL/min. Oven and tray temperatures were kept at 40°C. Mass spectra were acquired over the scan range m/z 300—2000 in the negative mode. Heated Electrospray Ionisation Ion Trap ionized the separated oligomers in an LTQ Velos Pro Mass Spectrometer (UHPLC-ESI-IT-MS) coupled to the UHPLC. The ratio of triGalA with or without methyl esterification was calculated from the peak area.

2.8. Determination of degree of methyl-esterification

Pectin samples (5mg) were saponified using 1 ml of 0.1M NaOH for 24 h (1 h at 4 °C, followed by 23 h incubation at RT). To the pectin blank, 1 ml of ultra-pure water was added. The head-space vials were immediately sealed with a Teflon-lined rubber septum. To determine the degree of methyl-esterification (DM) a gas chromatography method was used as previously described [36].

2.9. Determination of degree of blockiness

The degree of blockiness (DB) is calculated as the number of GalA residues present as non-methyl-esterified mono-, di- and triGalA released by endo-polygalacturonase related to the total amount of non-methyl-esterified GalA residues present and expressed as a percentage [29,37,38]. The amount of mono-, di- and triGalA after the PG/PL digestion of pectins was determined by HPAEC-PAD. For the quantification GalA, GalA2 and GalA3 were used. Since the alkaline elution conditions remove all methyl esters from the oligo-uronides, no distinction could be made between methyl-esterified and non-

methyl-esterified GalA3. The amount of GalA31 (1 methyl ester) as measured by HILIC-ESI-IT-MS was used to calculate the amount of non-esterified GalA3. DB was calculated using the following formula:

$$\mathbf{DB} = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_{n}\text{released}]_{\text{non-esterified}} \times n}{[\text{total non-esterified GalA in the polymer}]} \times 100$$

2.10. Reporter cell assays

To study whether pectins can activate TLRs or inhibit TLRs, activation or inhibition assays were performed with pectins using HEK-BlueTM cells expressing human TLRs (Invivogen). HEK-BlueTM hTLR cells were seeded in 96 wells plates at the indicated concentrations (Table 2) in 180 µl/well and were incubated overnight. The next day, the DMEM medium was replaced by DMEM medium containing pectins in the concentrations 0.5 mg/ml, 1 mg/ml or 2 mg/ml. Experiments to compare lemon and orange pectins were tested at 1 mg/ml only. Activation of the TLRs was studied by treating the cells with the pectins for 24 hours. Inhibition of the TLRs was studied by pre-treating the cells with pectins for 1 hour followed by addition of 20 µl of the TLR specific agonist (Table 2). Culture medium was used as negative control and the TLR specific agonist was used as positive control for 24 hours (Table 2). After 24 hours of incubation, media supernatant was mixed with QUANTI-Blue (Invivogen) in a ratio of 1:10. After 1 hour of incubation, NF-κB activation was quantified at 650 nm using a Versa Max ELISA plate reader (Molecular devices, Sunnyvale, CA, USA). Incubation steps were performed at 37°C and 5% CO2. TLR activation data were represented as fold change compared to negative control. TLR inhibition data were represented as fold change compared to the positive control. Each experiment was performed at least five times.

Table 2. *Reporter cell seeding density and their agonists*

Cell line (Invivogen)	Cell density for seeding	Agonist (Invivogen)
HEK-Blue hTLR2 (also expresses TLR1 and TLR6)	2.8 * 10^5 cells/ml (180 ul/well)	TLR2: Heat Killed Listeria Monocytogenes (10^7 cells/ml) TLR2/1: PAM3CSK4 (10 ng/ml) TLR2-6: FSL-1 (100 ng/ml)
HEK-Blue hTLR3	2.8 * 10^5 cells/ml (180 ul/well)	Poly-inosinic-olycytidylic acid (low molecular weight) (5 µg/ml)
HEK-Blue hTLR4	1.4 * 10^5 cells/ml (180 ul/well)	Escherichia coli K12 Lipopolysaccharide (10 ng/ml)
HEK-Blue hTLR5	1.4 * 10^5 cells/ml (180 ul/well)	Salmonella typhimurium derived flagellin (10 ng/ml)
HEK-Blue hTLR7	2.2*10^5 cells/ml	Imiquimod (5 mg/ml)
HEK-Blue hTLR8	2.2*10^5 cells/ml	Single stranded RNA (ssRNA40/LyoVecTM, 2 µg/ml)
HEK-Blue hTLR9	4.5 * 10^5 cells/ml (180 ul/well)	Type B CpG oligonucleotide (ODN2006; 0.25 µM)

2.11. Protein immunoprecipitation and ELISA for binding of TLR2 to pectin

hTLR2-HA protein was isolated from HEK 293/hTLR2-HA (Invivogen) as described before [13]. HA-tagged proteins were immunoprecipitated using Pierce® anti-HA agarose (Thermo Scientific, Waltham, MA, USA). The proteins were eluted using 50 µg/ml HA peptide (Thermo Scientific) for 30 min at 30°C. HA peptide was removed from the protein sample by using Zeba Spin Desalting Columns and Devices, 40K MWCO (Thermo Scientific). Protein concentration was quantified using BCA protein assay kit (Thermo Scientific).

To confirm that specific pectins bind to TLR2, a capture ELISA was performed as described before [13]. Isolated TLR2-HA was applied in concentrations 0.1 µg, 1 µg and 10 µg/well. For each pectin, rat-anti pectin antibody LM20 (1:100; Plantprobes, Leeds, UK) was used as positive control for pectin binding, to confirm even pectin immobilization. Each experiment was performed at least five times.

2.12. TLR2/1 inhibitory effect of pectins on IL-6 and IL-10 production

In addition to the TLR2/1 inhibition assay on reporter cell lines, TLR2/1-dependent inhibition of immune responses by pectins was also tested on THP-1 cells differentiated to macrophages [39]. THP-1 cell differentiation was induced by stimulation of THP-1 cells (1x106 cells/ml) with 100 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma) in a 12 wells plate (in 0.5 mL medium) for 48 hours at 37°C and 5% CO2. The adherent cells were washed with PBS (Westburg, Grubbenvorst, the Netherlands) to remove PMA. Next, they were treated with pectins at 100 µg/ml dissolved in culture media. This concentration of pectins has previously been shown to be effective in activating and inhibiting macrophage responses [13]. Non-treated THP-1 cells were used as negative control. After 1 h of pre-treatment with the pectins, 10 ng/ml of Pam3CSK4 was added. THP-1 cells treated with Pam3CSK4 or pectin only were used as control. After 24 h incubation, media supernatant was collected. IL-6 and IL-10 were quantified in the supernatant by ELISA according to the manufacturer's protocol (eBioscience, San Diego, USA).

2.13. Statistical analysis

The results were analyzed using Graphpad Prism program (La Jolla, CA, USA). Normal distribution was confirmed using the Kolmogorov-Smirnov test. Data that were not normally distributed were log transformed before analysis. Statistical comparisons were performed using two-way ANOVA was performed. Post-testing was performed with Tukey to test statistical differences between vehicle and pectins (* $p < 0.05$ was considered as statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), **** $p < 0.0001$) or to test statistical differences between week 1 and week 4 (# $p < 0.05$ was considered as statistically significant; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$), #### $p < 0.0001$). Values are expressed as mean ± standard error (SD).

3. Results and discussion

3.1. Chemical composition of pectins

Pectins obtained from lemon and orange were characterized for the degree (percent) of methyl-esterification (DM), molecular weight, and sugar composition. The degree of blockiness was calculated after enzymatic fingerprinting of the pectins and subsequent analysis of the released oligosaccharides by HPAEC and HILIC-MS. The characteristics of the pectins are given in Table 3. The type of citrus peel and the industrial processing conditions resulted in only small differences in the GalA content, the content and composition of neutral sugars, and the molecular weight of the pectins. The molecular weight distribution of pectins (Figure S1) does not show major differences between the pectins. The orange pectins were characterized by a DM of 32 and 64 with a DB of 35 and 37, respectively. The methyl-esterification of the eight lemon pectins ranged from DM18 to DM88, whereas the blockiness varied between DB31 and DB93. DB is representing the charge density rather than the total charge of the molecule. For the two lemon pectins having a DM of 32 and 64, the (similar) DB values indicate that for both pectins, about 35% of the non-esterified GalA residues are present in blocks, although the total number of non-esterified GalA residues differ. For the lemon DM52 and DM88 pectins, the distribution of the non-esterified GalA residues over blocks is quite different as they have respectively 33% and 71% of the non-esterified GalA residues distributed in blocks. Thus, the DB allows us to recognize different methyl ester distributions of pectins, even when the level of methyl esterification is similar.

Table 3. *Structural characteristics of pectins. Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample. Degree of blockiness (DB): the amount of mono-,di- and triGalA per 100 mol of the non-esterified GalA in the sample. Molecular weight (Mw) as measured by HPSEC. Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, UA = Uronic Acid.*

Pectin	origin	DB (%)	Mw (kDa)	Sugar composition (mol%)					Carbo-hydrate content (w/w%)*
				Rha	Ara	Gal	Glc	UA	
DM32	orange	35	77	1	3	6	1	85	57
DM64	orange	37	92	1	7	8	2	82	81
DM18	lemon	86	78	1	0	2	0	97	62
DM19	lemon	94	75	1	1	3	0	95	63
DM33	lemon	48	70	1	0	6	0	93	80
DM43	lemon	60	79	0	0	0	0	99	77
DM49	lemon	33	114	0	1	2	0	96	73
DM52	lemon	31	74	1	3	6	0	89	80
DM84	lemon	71	113	1	5	6	1	87	65
DM88	lemon	91	91	1	3	5	0	91	67

3.2. TLR2 is activated by high DM orange pectins while TLR2/1 is inhibited in a DM-independent manner by orange and lemon pectins

Pectins might influence immunity through Toll-like receptor (TLR) signaling [12]. It has been shown for lemon pectins that the magnitude by which lemon pectin impact TLRs depends on the DM [12,13]. It is unknown whether other structural different pectins have similar DM dependent effects on TLR signaling. Therefore, the TLR activating and inhibiting effects of two orange pectins with a DM value of 32 and 64 with that of lemon pectins with a DM value of 33 and 52 were compared (Figure 2). This was done by using HEK-BlueTM cells expressing either TLR 2, 3, 4, 5, 7, 8, or 9.

As shown in Figure 2, TLR2 was activated, whereas TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 were not activated by any of the pectins. TLR2 was specifically activated by orange pectin while lemon pectins did not have any TLR2 activating capacity. This TLR2-activation by orange pectin was DM dependent as high DM orange DM64 pectins activated TLR2 by 5.3-fold ($p < 0.0001$) while DM32 did not have such an effect.

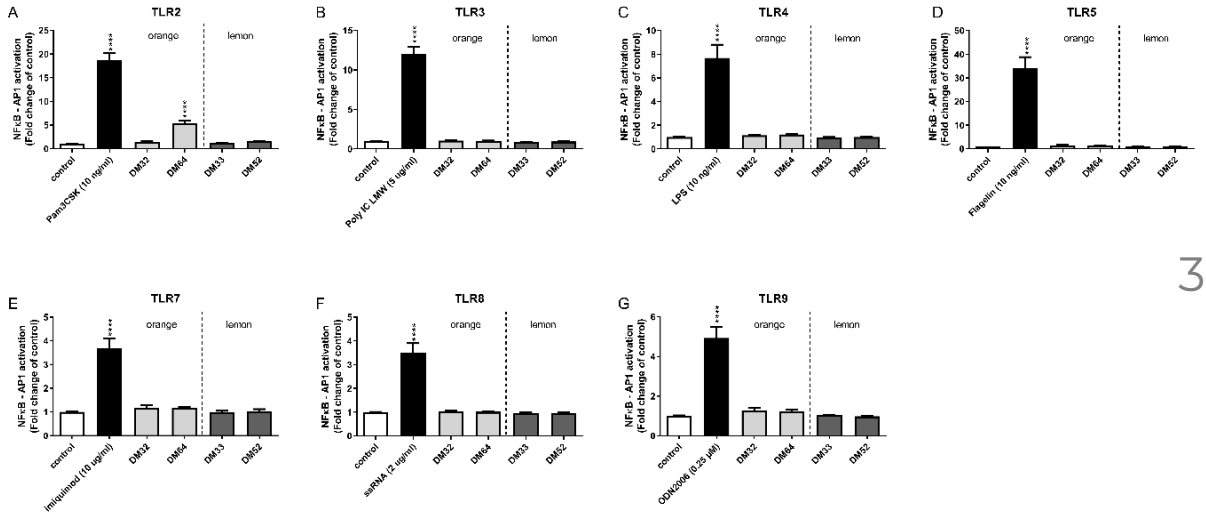

Figure 2. *TLR activation after stimulation of TLR-expressing reporter cells with orange and lemon pectins with a different DM. Activation of TLR2 (A), TLR3 (B), TLR4 (C), TLR 5 (D), TLR 7 (E), TLR8 (F), TLR9 (G) by orange and lemon pectins. The statistical differences between control and pectin samples were quantified using the one-way ANOVA test (**** p < 0.0001) (n=9).*

As shown in Figure 3, the used sets of orange and lemon pectins have a different TLR inhibiting capacity. For the inhibition studies, TLR2/1 was studied by using Pam3CSK4 as agonist, TLR2-6 by using FSL-1 as agonist, and to study total TLR2 inhibition the agonist HKLM was used. Both lemon pectin and orange pectin specifically inhibited TLR2/1 and had no inhibitory effects on FSL-1 and HKLM induced TLR2 activation. Orange pectins did not inhibit TLR2/1 in a stronger way with gradual lower DM content, which is the opposite of what was observed before with lemon pectins [13]: orange pectin with a DM64 had a higher inhibiting effect on TLR2/1 than orange pectin with a DM32 (50.0 ± 0.05%, $p < 0.0001$ vs 40.4 ± 0.05%, $p < 0.0001$, respectively). Lemon pectin inhibited just as reported before TLR2/1 stronger with lower DM. Lemon DM33 had a stronger inhibitory effect (44.3 ± 0.05%; $p < 0.0001$) than lemon pectin with a DM of 52 (14.6 ± 0.05%; $p < 0.05$). In addition to these opposite DM-dependent effects of orange and lemon pectin on TLR2/1, differences in inhibition of other TLRs were observed. Orange DM32 pectin inhibited TLR3 and TLR8 (TLR3: $p < 0.0001$; TLR8: $p < 0.001$) while orange DM64 pectin also inhibited TLR3, TLR5, TLR8 and TLR9 (TLR3: $p < 0.0001$; TLR5: $p < 0.001$; TLR8: $p < 0.001$; TLR9: $p < 0.05$). Lemon DM33 pectin had in addition to inhibition of TLR 2/1 ($p < 0.0001$) no other TLR inhibitory effects. However, lemon DM52 pectin inhibited in addition to TLR2/1 also TLR3 and

TLR4 (TLR2/1: $p < 0.05$; TLR3: $p < 0.001$; TLR4: $p < 0.001$). None of the tested pectins did inhibit TLR2, TLR2-6, and TLR7. These findings suggest that the tested orange and lemon pectins have different inhibitory capacity towards the various TLRs, although both types do inhibit TLR2/1.

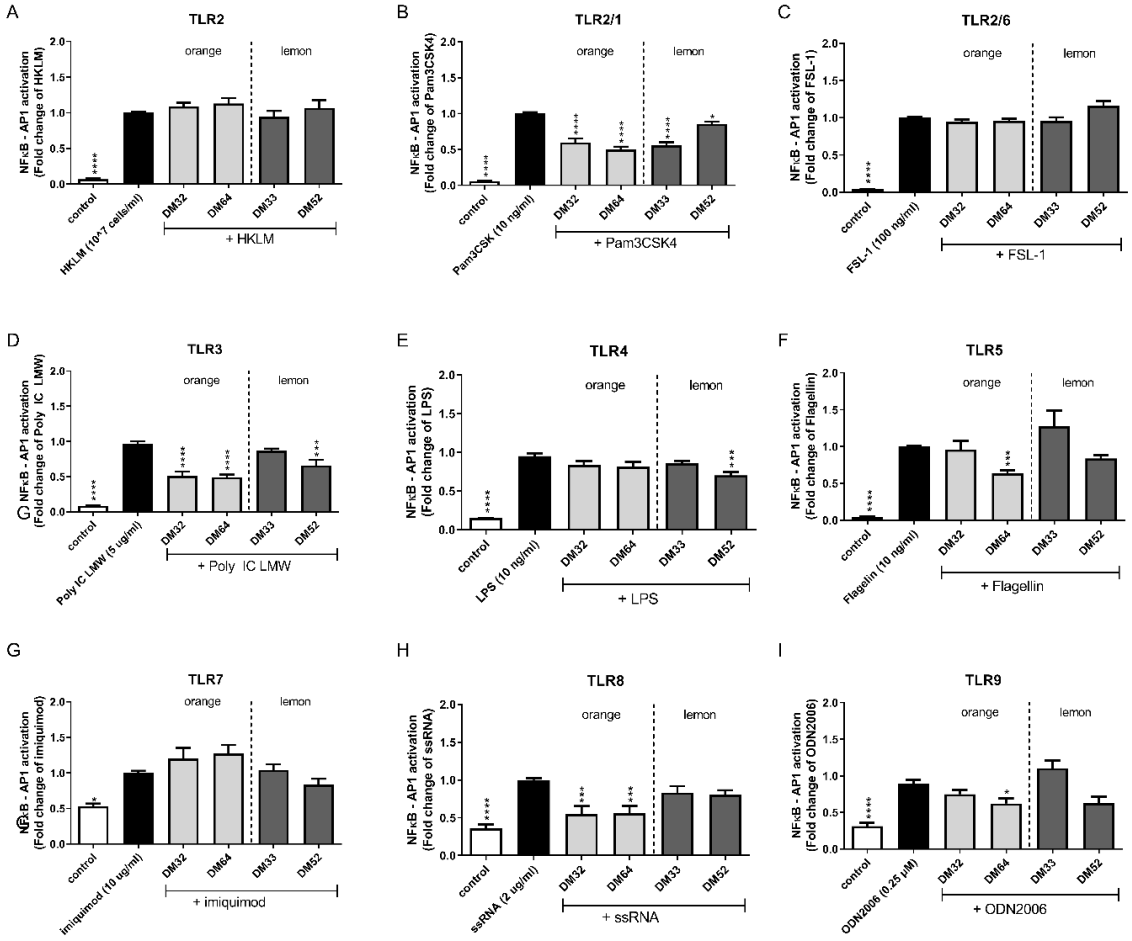

***Figure 3**. Inhibition of TLRs after stimulation with orange and lemon pectins with different degrees of methyl-esterification (DM). Inhibition of TLR2 (A), TLR2/1 (B), TLR2-6 (C), TLR3 (D), TLR4 (E), TLR5 (F), TLR7 (G), TLR8 (H), TLR9 (I) by orange and lemon pectins. HEK-BlueTM hTLR cells were first pre-incubated for 1 hour with pectins (1 mg/ml) and subsequently stimulated with the TLR-specific agonist. The statistical differences between TLR ligand and pectin samples were quantified using the one-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$) (n=9).*

3.3. Pectin's degree of blockiness has overarching effects on DM induced effects on TLR2/1 inhibition

Here and in a previous study, it has been demonstrated that TLR2/1 inhibition by lemon pectins was DM dependent with more pronounced inhibition of lower DM pectins (Sahasrabudhe et al., 2018). As orange pectins, with other structural features, did not seem to have this same DM dependent inhibitory effects on TLR2/1 with lowering of DM, it was questioned whether other structural properties of pectins may play a role in TLR2/1 inhibition. In search of such differences, the degree of blockiness (DB) of the tested orange and lemon pectins was determined (Table 3). Orange DM32 pectin has a lower DB than the lemon DM33 pectin (35% and 48%, respectively). Furthermore, orange DM64 pectin has higher DB than the lemon DM52 pectin (37% and 31%, respectively). To visualize the impact of the DB more clearly the TLR2/1 inhibiting capacity was expressed according to the variations in DB (Figure 4). Orange pectin with a DB of 37 compared to lemon pectin with a DB of 31, but rather similar DM, resulted in more inhibition of TLR2/1 (35,4% $p < 0.0001$). Additionally, orange pectins with a DB of 35 compared to lemon pectins with a DB of 48, but similar DM, did not result in significant differences in TLR2/1 inhibition. These results suggest that pectins with a high DB induce more TLR2/1 inhibition, but the strength of this DB dependent TLR2/1 inhibition seems to be dependent on the DM of pectins as DB-dependent effects on TLR2/1 inhibition were stronger in high DM pectins than by low DM pectins. This may be related to the different structural patterns of low and high DM pectins with a high DB (Figure 1). Low DM pectins with either a high or low DB contain larger blocks of non-esterified GalA acid residues than pectins with a higher DM and high DB. Furthermore, low DM pectins with a low and high DB both have large blocks of non-esterified GalA residues, while high DM pectins have a large difference in block size between low and high DB pectins (Figure 1). This can be explained by the higher percentage of non-esterified GalA residues in low DM pectins (Daas et al., 2000). To study this in more detail, lemon pectins were used that varied in DM and DB with the aim to study which combination of the structural parameters DM and DB is responsible for the most pronounced TLR2/1 inhibition. This was not possible with orange pectin as these pectins could not obtained with the same variation in DM and DB.

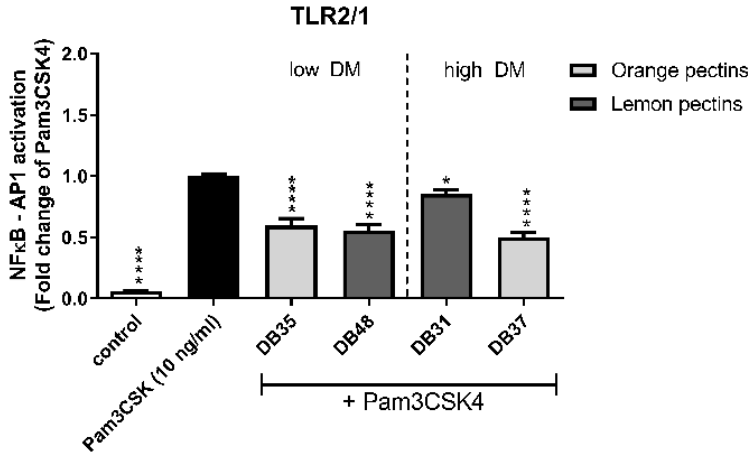

Figure 4. *Inhibition of TLRs after stimulation with orange and lemon pectins with different degrees of blockiness (DB). Inhibition of TLR2/1 by orange and lemon pectins with a different degree of blockiness. HEK-BlueTM hTLR cells were first pre-incubated for 1 hour with pectins (1 mg/ml) and subsequently stimulated with the TLR-specific agonist. The statistical differences between TLR ligand and pectin samples were quantified using the one-way ANOVA test (* p < 0.05, *** p < 0.001 and **** p < 0.0001) (n=9).*

The TLR2/1 inhibitory capacity of six lemon pectins was compared. The pectins could be grouped into three levels of similar DM of 19%, 46% or 86%, but with a different degree of blockiness (Table 3). For all three levels of DM there was one pectin with a lower degree of blockiness (DM18, DM49, DM84) and one with a higher degree of blockiness pectin (DM19, DM43, DM88) available. The pectins were first tested on TLR2/1 inhibiting and TLR2 activating capacities. For TLR2/1 inhibition there were clear DB dependent effects (Figure 5). All high DB pectins together induced more inhibition of TLR2/1 than all low DB pectins together ($p < 0.01$). However, the magnitude by which the DB inhibited TLR2/1 was dependent on the DM. With low DM pectins, both high DB pectin (DM19) and low DB pectin (DM18) inhibited TLR2/1 strongly (69.1 ± 0.08%, $p < 0.0001$ vs Pam3CSK4 and 63.2 ± 0.08%, $p < 0.0001$ vs Pam3CSK4, respectively), but not significantly different. For intermediate DM pectins, the high DB pectin (DM43) inhibited TLR2/1 significantly ($p < 0.05$) stronger than the low DB pectin (DM49) (71.9 ± 0.07%, $p < 0.0001$ vs Pam3CSK4 and 58.0 ± 0.07%, $p < 0.0001$ vs Pam3CSK4) illustrating the impact of DB. The high DB pectin (DM43) did not inhibit TLR2/1 significantly less strong than the DM18 and DM19 pectins, indicating that pectins with a higher DM, but also a higher DB can reach similar levels

of TLR2/1 inhibition as low DM pectins. Furthermore, the very high DM pectin with a high DB (DM88) did not inhibit TLR2/1 stronger than the very high DM pectin with a low DB pectin (DM84). Both high and low DB pectins with a high DM did not inhibit TLR2/1 as strong as the pectins the lower DM pectins (DM < 84) (44.1 ± 0.07% vs Pam3CSK4 and 37.5 ± 0.07%, $p < 0.0001$ vs Pam3CSK4) suggesting that probably above a certain DM threshold the DB does not enhance TLR2/1 inhibitory capacity anymore. Overall the results demonstrate that high DB pectin can inhibit TLR2/1 stronger than low DB pectins. This implies that the blockwise distribution of non-esterified GalA residues (high DB) of pectins with an intermediate DM can reach a similar level of TLR2/1 inhibition as low DM pectins. The effects of DB were most visible in intermediate DM pectins (DM43-49) and not in very low DM (DM18-19) or very high DM (DM84-88) pectins. None of the six pectins activated TLR2 (Figure S2).

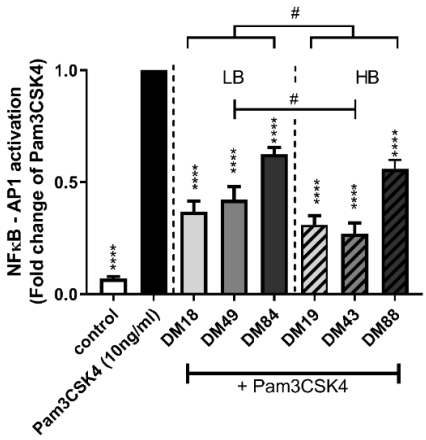

Figure 5. *Impact of the methyl-ester distribution (DB) of lemon pectins on inhibition of TLR2/1. Inhibition of Pam3SCK4 induced TLR2/1 activation by DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin in the concentration 2.0 mg/ml. The statistical differences between Pam3SCK4 and pectin samples were quantified using one-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$). Statistical differences between LB and HB pectins were tested by repeated measures two-way ANOVA (# $p < 0.05$) (n=6).*

3.4. Impact of DB and DM on binding to the TLR2 protein

To further substantiate the DB-dependent binding of pectin to TLR2 a capture ELISA was performed that measures the direct binding of pectins to TLR2. This approach allows us to determine true binding of pectin by the

TLR2 receptor rather than neutralizing the agonist. All high DB pectins showed stronger binding to TLR2 than the low DB pectins ($p < 0.05$) (Figure 6). This effect was concentration-dependent and most pronounced with 10 µg TLR2 protein (Figure S3). However, similar to what was observed in the TLR2/1 inhibition assay, the DB-induced effects were dependent on the DM of the pectins. Both pectins with DM18 and DM19 having either a low and high DB showed the strongest binding to TLR2 (both $p < 0.0001$ vs control). The degree of binding between those pectins was similar and not significantly different, indicating that the difference in DB did not induce a difference in TLR2 binding at low DM. At an intermediate DM, high DB pectin (DM43) showed significantly stronger binding to TLR2 than low DB (DM49) pectin (Fig. 6; $p < 0.05$). The high DB pectin bound as strong as low DM pectins to TLR2. At a very high DM, there is no significant difference between the high and low DB pectins measured in TLR2 binding. Together, these findings suggest that pectins with blockwise distributed non-esterified GalA residues bind stronger to TLR2 than pectins with randomly distributed non-esterified GalA.

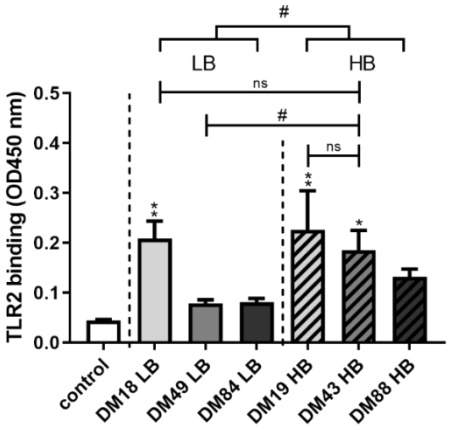

Figure 6. *LB and HB pectins bind differently to TLR2. TLR2 binding to DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin. The statistical differences between control and pectin samples were quantified using the one-way ANOVA test (* $p < 0.05$ and ** $p < 0.01$). Statistical differences between LB and HB pectins were also quantified using repeated measures two-way ANOVA test (# $p < 0.05$) (n=9). Ns = not significant.*

3.5. Pectin inhibited TLR2/1 induced IL-6 secretion, but not the TLR2/1 induced IL-10 secretion in macrophages

The possible inhibiting effect of pectin's DM and DB on TLR2-TLR1 induced inflammatory responses was also investigated using THP-1 differentiated human macrophages. The cells were incubated with or without the TLR2/1 stimuli Pam3CSK4 in presence of the six pectins with a low DB and a high DB. The secretion of the pro-inflammatory IL-6 and anti-inflammatory IL-10 were quantified (Figure 7). The pectins, without Pam3CSK4 stimulation, did not stimulate IL-6 or IL-10 secretion in the THP-1 differentiated macrophages. However, all pectins did significantly inhibit IL-6 secretion ($p < 0.0001$ vs Pam3CSK4) in Pam3CSK4 stimulated macrophages, but they did not inhibit TLR2/1 induced IL-10 secretion.

The inhibitory effects of the pectins on IL-6 secretion corresponded with the trends of inhibition of TLR2/1 as was observed with the same pectins in the TLR2 reporter cell line. The low DM pectins (DM18 and DM19) did inhibit TLR2/1 strongly. Also, intermediate DM pectins combined with a high DB (DM43) did inhibit TLR2/1 induced IL-6 secretion stronger than intermediate DM pectin with a low DB pectin (DM49; $p < 0.01$). Very high DM pectins did not show this difference in TLR2/1 induced IL-6 secretion between low and high DB pectins corresponding to the inability to suppress TLR2/1 in the reporter cell lines. These results show that the pectins not only inhibit TLR2/1 signaling, but also the subsequent initiation of pro-inflammatory IL-6 secretion after TLR2/1 stimulation with Pam3CSK4. The pectins with a blockwise distribution of non-esterified GalA residues were most effective in inhibiting TLR2/1 induced IL-6 secretion.

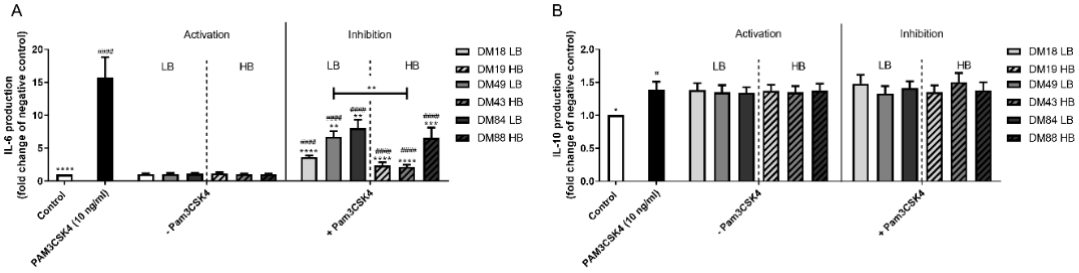

Figure 7. *IL-6 and IL-10 secretion by THP-1 macrophages stimulated with LB and HB pectins in presence or absence of Pam3CSK4. THP-1 macrophages were stimulated with pectins in presence or absence of Pam3CSK4. The statistical differences between the Pam3CSK4 and pectin samples test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$) or between control and pectin samples test (#### $p < 0.0001$) were quantified using the two-way ANOVA test. (n=5).*

4. Discussion

Several studies have shown the protective effects of pectins on the development of mucositis, pancreatitis, diet-induced obesity or autoimmune diabetes in mouse models [13,40–42]. The exact mechanisms responsible for these protective effects of pectins are not fully understood. One of the mechanisms by which pectin can protect against inflammatory disease is by modulating TLR signaling [12,13,24]. This modulation of TLRs depends on structural parameters of pectins, such as the DM [12,13]. However, the impact of other structural features such as the blockwise distribution of non-esterified GalA residues was so far unknown. Here, it was shown that the DB is an essential factor in the attenuating effects of pectins on TLR2/1 signaling and that the effects of the DB are most distinct in pectins with higher DM.

The current study shows that pectins strongly inhibit TLR2/1, whereas other extracellular or intracellular TLRs are inhibited to a much lower extent by pectins. This seems in contrast to other studies which showed inhibition of TLR2-6, 4 or TLR9 induced immune responses by pectins [24,43]. However, the inhibitory effects of those pectins may be related to the presence of RG-I and RG-II side chains, which are almost absent in pectins from the current study. These pectins are mainly homogalacturonan pectins [24,43]. Sahasrabudhe et al. also confirmed that homogalacturonan pectins inhibit TLR2/1 specifically and not TLR2-6, TLR4, or TLR5 [13]. Sahasrabudhe et al. provided evidence that homogalacturonan pectins interact with the TLR1 binding site on TLR2, preventing dimerization of TLR2/1 [13]. The homogalacturonan pectins in that study were not able to inhibit dimerization of TLR2-6, which corroborates the current findings. Together, the current findings show that homogalacturonan pectins are very specific in inhibiting TLR2/1 immune responses, whereas RGI and RGII pectins can inhibit other TLR mediated immune responses [24,43].

Our data illustrate that the high DB strengthens the DM-dependent TLR2/1 inhibition. This suggests that not only the high level but also the blockwise distribution of non-esterified GalA residues of pectins (Figure 1) is important for TLR2/1 inhibition. This is confirmed by the observation that both low DM pectins but also intermediate DM pectin with a high DB, which have a more blockwise distribution of their non-esterified GalA residues, inhibited TLR2/1 strongly. This argumentation is further supported by the observation that intermediate DM pectin with a low DB, having a more random distribution of its non-esterified GalA residues, inhibited TLR2/1 less efficiently. However, the very high DM pectins, which showed very low inhibition of TLR2/1, contain a

very low number of non-esterified GalA residues [29]. Together, these findings suggest that the blockwise distribution of non-esterified GalA residues in pectins induces more inhibition of TLR2/1 than in pectins with a more random distribution of non-esterified GalA residues or having a very low number of non-esterified GalA residues.

The reason that a high DB in very high DM pectins is not leading to a significant inhibition, could be simply due to the fact that there is a limited number of non-esterified GalA residues present, despite that these non-esterified GalA residues are blockwise distributed. The block size of DM88 pectin might be too small to induce a strong inhibition of TLR2/1, whereas the larger blocks in DM19 and DM43 pectins still are inhibitory. In general, a DB-value does not provide information on whether the corresponding pectin may contain one big block or several smaller blocks of non-esterified GalA residues [38,44]. Based on the absolute number of non-esterified GalA residues present, DM19 and DM43 contain certainly more blocks than DM88 pectin. This suggests that a combination of block size and distribution [38,44] may be involved in TLR2/1 inhibitory capacity of pectins.

Next, the binding of low DB and high DB pectins to TLR2 was investigated to confirm the true binding of pectins to the receptor rather than to the agonist. The binding of pectin to the receptor was confirmed and the DB-dependent patterns of binding were similar to what was observed for TLR2/1 inhibition in the reporter cell lines. Furthermore, in this capture ELISA less binding was observed for very high DM pectins which confirms the aforementioned reasoning that blockwise distribution and block-sizes of non-esterified GalA residues are important for the capacity of pectins to bind to TLR2 and preventing TLR1 to associate. In addition, it was confirmed that a more blockwise distribution of non-esterified GalA residues bind stronger to TLR2 than randomly distributed non-esterified GalA residues. This binding may be established through ionic binding between the blocks of non-esterified GalA residues and TLR2. Ionic binding has been shown to play an important role in the interaction of pectins and TLR2 [45]. More negatively charged pectins (low DM) bound stronger to TLR2 than less negatively charged pectins (high DM). The binding between negatively charged pectins became stronger to mutant TLR2 proteins with more positively charged amino acids [13]. Pectins with a blockwise distribution (high DB) of non-esterified GalA residues have larger areas with a negative charge (higher charge density) compared to random distributed non-esterified GalA residues [46]. This suggests that the larger negative charge areas of the

blockwise distributed non-esterified GalA residues in pectins may be of importance in the binding of pectins to TLR2.

The current study also showed that high DB pectins were more effective in suppressing TLR2/1 induced IL-6 responses than low DB pectins, which is in line with the TLR2/1 inhibition as was observed in TLR2/1 inhibition assays. This suggests that pectins not only affect TLR2/1 signaling, but also the subsequent initiation of IL-6 secretion. Inhibition of IL-6 responses may be beneficial under inflammatory conditions, as high levels of IL-6 play an important role in intestinal inflammation [47,48]. The secretion of IL-6 strongly depends on TLR2 activation [49,50]. This has been observed in mice with mucositis in which high activation of TLR2 induces inflammation characterized by high IL-6 levels [51,52]. Low DM pectins were able to reduce this inflammatory response in mucositis by inhibiting TLR2 signaling and IL-6 secretion [13]. As HB pectins were able to inhibit the TLR2/1 induced IL-6 secretion, they may also serve as a dietary component with potential anti-inflammatory effects on mucositis.

5. Conclusion

In the current study, we hypothesized that the level and distribution of methyl-esters in pectin determine the efficacy of pectins to impact TLR signaling. The current study demonstrates that the high number and blockwise distribution of non-esterified GalA residues in pectins are responsible for the TLR2/1 inhibitory effects. Such pectin structures were most effective in preventing the induction of pro-inflammatory cytokine responses in human macrophages. This knowledge is important for a better understanding of the structural characteristics of pectins with TLR2 inhibiting properties and can be instrumental in the design of functional food applications with strong TLR2-blocking properties. Consumers may benefit from consuming pectins with a high DB as the blockwise distribution of non-esterified GalA residues in those pectins may limit the development of small intestinal inflammation induced by high activation of TLR2 (Kaczmarek et al., 2012; Sahasrabudhe et al., 2018), whereas pectin may stimulate microbial-derived SCFA production in the colon (Tian et al., 2016). Ultimately, understanding which specific pectin structures protect the intestinal immune barrier may contribute to the prevention of the development of immune-related disorders.

Acknowledgments

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Appendix

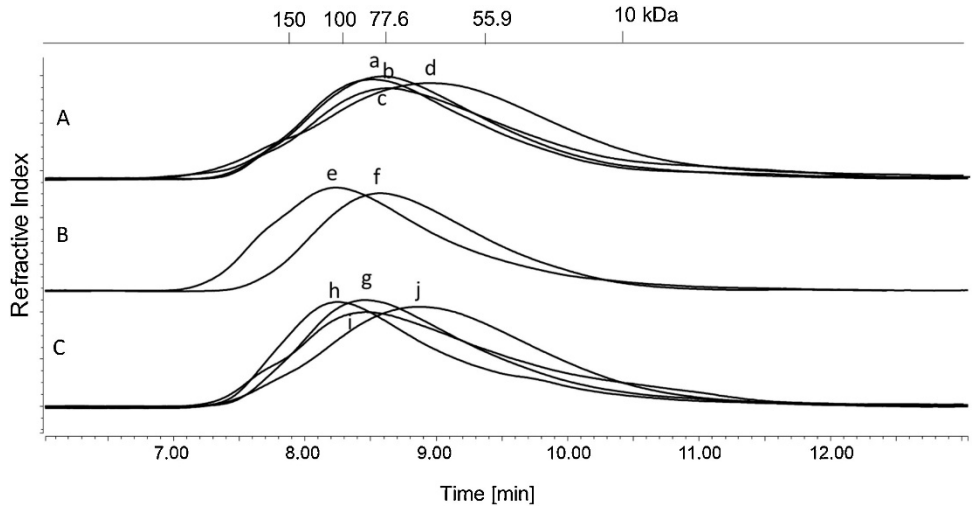

***Figure S1**. HPSEC elution patterns of the pectins. Molecular weights of pectin standards (in kDa) are indicated. A = low DM pectins, B = intermediate DM pectins, C = high DM pectins. a = DM32, b = DM18, c = DM19, d = DM33, e = DM49, f = DM43, g = DM64, h = DM88, i = DM84, j = DM52.*

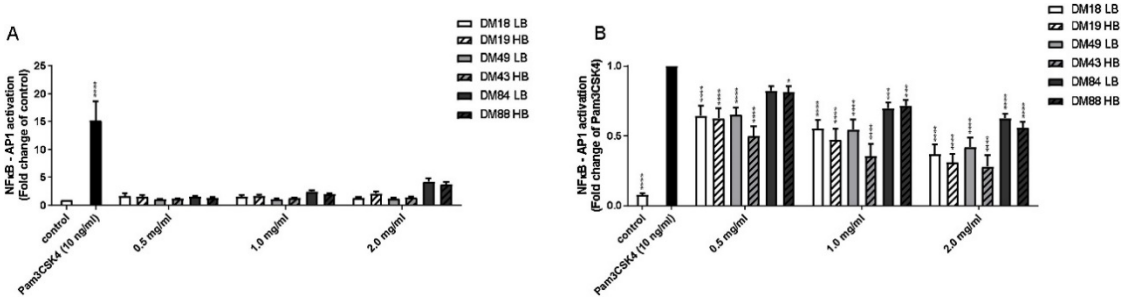

Figure S2.** Impact of the DB of pectins on TLR2 activation and TLR2/1 inhibition in a concentration dependent manner. TLR2 activation (A) and TLR2-1 induced inhibition (B) by DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin in the concentrations 0.5, 1.0 and 2.0 mg/mL. The statistical differences between control and pectin samples (A) or Pam3CSK4 and pectin samples (B) were quantified using the two-way ANOVA test (* $p < 0.0001$) (n=6).*

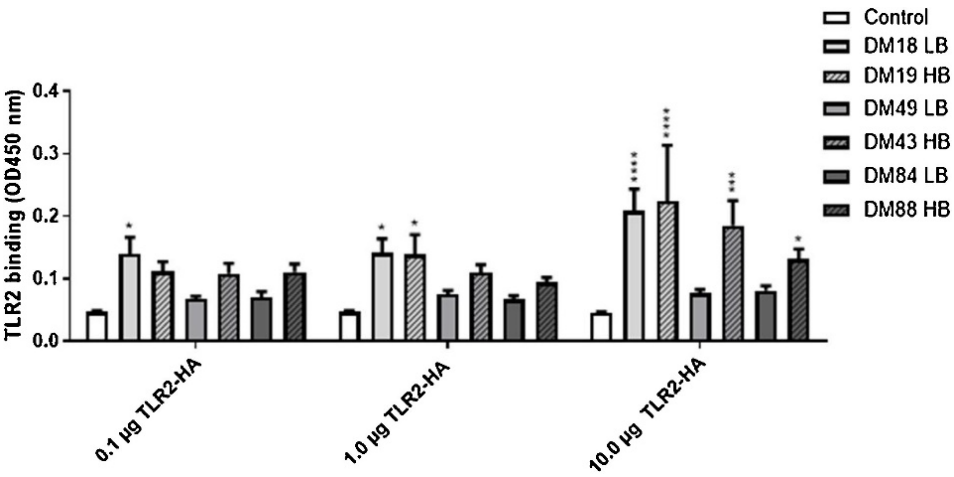

Figure S3. *LB and HB pectins bind to TLR2 in a concentration dependent manner. TLR2 binding to DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin. The statistical differences between control and pectin samples were quantified using the two-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$) (n=9).*

Chapter 4

TLR 2/1 interaction of pectins depends on its chemical structure and conformation

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Abstract

Pectins have demonstrated health benefits through direct interaction with Toll-like receptor 2 (TLR2), although the underlying structure-function relationships are still unknown. Pectin polymer interactions with TLR2 were studied. Molecular docking analysis was performed using two GalA heptamers, $GalA_7Me^{1,7}$ and $GalA_7Me^{2,5}$ in which molecular relations were measured and simulated in various possible conformations. Pectins were characterized by enzymatic fingerprinting to determine their methyl-ester distribution patterns. The response of 12 structurally different pectic polymers on TLR2 binding and the molecular docking with two pectin oligomers clearly demonstrated interactions with TLR2 in a structure-dependent way, where blocks of (non)methyl-esterified GalA were shown to inhibit TLR2/1 dimerization. Our results help to recognize molecular interactions between pectins and TLRs and may be used to understand the immunomodulatory effects of certain pectins via TLR2. Knowledge of how pectins with certain methyl-ester distribution patterns bind to TLRs may lead to tailored pectins to prevent inflammation.

1. Introduction

The health effects associated with dietary fibers are more and more discussed in the literature, but mechanisms that could explain the effects are often still lacking. An obvious reason for that is the high diversity of dietary fibers in their structure and functionality. Moreover, dietary fibers used in research are often compared without appropriate characterization, causing numerous contradictions in the literature regarding their health effects [1, 2]. Some dietary fibers may play an important role in gut health by serving as fermentation substrates and energy sources for the gut microbiota [3, 4]. Upon fermentation, the microbiota will generate short-chain fatty acids (SCFAs) that, among other effects, may reduce inflammation by increasing the number of immunoregulatory cells in the gut [5, 6]. Nevertheless, beneficial effects of polysaccharides independently from SCFAs have been also reported [7, 8] including direct immune-modulating effects of fibers on immune cells [9].

Several *in vivo* and *in vitro* studies have been performed on the immunomodulatory effects of dietary fibers [1, 10-12]. A large variety of different plant-derived polysaccharides such as glucans, mannans and pectins have been studied for their immune system activating and -inhibiting properties [13]. The direct interaction of dietary fibers and the intestinal cells happens through interaction with the so-called pattern recognition receptors (PRRs) [14]. PRRs play a significant role in intestinal immune regulation, since they are responsible for recognizing exogenous molecules [2, 14]. Toll-like receptors (TLRs) form such a family of PRRs, which play an essential role in the activation of innate immunity [15] and proved to be involved in dietary fiber-induced immune signaling [16]. Dietary fibers have a highly complex and diverse structure and therefore they can either activate TLRs to different extents (e.g., high DM lemon pectin) [10], or inhibit TLR signaling and decrease intestinal inflammation (e.g., low DM lemon pectin) [11]. Through TLR signaling, fibers have shown to have several beneficial effects, including reduced intestinal permeability and thereby better gut barrier function [9, 10], promoting immune responses against pathogens [17] as well as reducing intestinal inflammation [11]. It has been demonstrated that the chemical differences such as the side chains and the chain length in fibers such as pectins can regulate immune effects [9, 17]. More information on the effect of the chemical structure of fibers on intestinal immunity is therefore important to understand and to predict the efficacy of dietary fibers [11, 17].

Pectin is a well-known soluble dietary fiber that has, both direct and indirect, nutritional and physiological health effects. Its biological properties have gained attention already for years [18]. Pectin is commonly used as functional

ingredient in the food industry due to its thickening and gelling capacity [19]. Commercial pectin is mainly composed of a linear chain of α-1,4 D-galacturonic acid (GalA) units, called homogalacturonan (HG), which covers approximately 70-90% of the pectin backbone and can be methyl-esterified at the GalA O-6 carboxyl group and, less commonly, be O-acetylated at the GalA O-2 or O-3 positions depending on the source [20]. Other domains of pectin are rhamnogalacturonan-I (RG-I) and RG-II. RG-I comprises 20–30% of GalA in of the pectin structure [21]. The technological and biological properties of a pectin depend on its structural characteristics like monosaccharide composition, level and distribution of methyl-esterification, level of acetylation, molecular weight (Mw), presence, type and length of side chains, and conformation/nanostructure or spatial structure [22-24]. The level and distribution of methyl-esters over the pectin backbone are fundamental elements contributing to pectin's functionality [10, 11, 21]. The percentage of methyl-esterified GalA residues over the backbone is defined as the degree of methyl-esterification (DM). The main methyl-ester distribution patterns are described as random or blockwise [25-28]. Non-esterified GalA distribution patterns were first defined by Daas et al. [28] as the degree of blockiness (DB) and absolute degree of blockiness (DB_{abs}) [25, 29]. DB is indicating the relative amount of non-esterified GalA residues present in PG degradable blocks, representing the distribution of non-esterified blocks in relation to the total of non-esterified GalA residues of the pectin molecule, while DB_{abs} is representing the distribution of non-esterified blocks over the entire pectin molecule. Other parameters describing also the methyl-esterified sequences over the backbone are degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme}) and degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}) [30]. DB_{abs} shows the fully non-esterified segments of the backbone, while DB_{PGme} and DB_{PLme} illustrate the different methyl-esterified sequences of the pectin degradable by PG or PL.

Sahasrabudhe et al. have shown that TLR2/1 is inhibited by lemon pectins in a DM-dependent manner, where a decreased DM increased TLR2/1 inhibiting and binding properties of pectins [11]. Furthermore, it has been observed that not only the level but also the distribution of methyl-esters determines the ability of pectins to influence TLR signaling, the more blockwise methyl-esterified the pectin is, the higher the TLR2/1 inhibitory effect [12]. However, pectins with a similar DM and DB might still have different sequences of non-esterified or methyl-esterified GalA residues [30]. It is not known whether such different sequences play a role in the interaction between TLR2 and pectins.

The aim of this study was to understand the relationship between pectin structure and conformation and TLR2/1 inhibition. To investigate the

structural characteristics of pectins underlying the binding and inhibition of TLR2/1, pectins with known TLR2/1 inhibiting capacities were extensively characterized by enzymatic fingerprinting methods for their level and distribution of methyl-esters. For the binding, molecular relations were measured and simulated in various possible conformations. Now, for the first time, we used docking analysis, which helped to recognize molecular interactions between pectins and TLRs and may be used to understand why only pectins with a certain structure bind to TLRs.

2. Materials and methods

2.1. Materials

Commercially extracted lemon (L) pectins L18 (DM18%), L19 (DM19%), L32 (DM32%), L43 (DM43%), L49 (DM49%) were provided by CP Kelco (Copenhagen, Denmark) and orange (O) pectins O32 (DM32%), O59 (DM59%), O64 (DM64%) were provided by Andre Pectin (Andre Pectin Co. Ltd., Yantai, China). Endo-polygalacturonase (Endo-PG, EC 3.2.1.15) from *Kluyveromyces fragilis* [28] and pectin lyase (PL, EC 4.2.2.10) of *Aspergillus niger* [31] were used to degrade the citrus pectins. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), VWR International (Radnor, PA, USA), or Merck (Darmstadt, Germany), unless stated otherwise.

2.2. Characterization of pectins

Determination of the neutral monosaccharide composition of citrus pectins was carried out by acid hydrolysis and neutral sugars released were derivatized and analyzed as their alditol acetates using gas chromatography. Galacturonic acid content of the hydrolysates was determined by the automated colorimetric *m*-hydroxydiphenyl method as previously described [30, 32, 33]. To determine the degree of methyl-esterification pectin samples were saponified using 0.1M NaOH for 24 h (1 h at 4 °C, followed by 23 h at room temperature). The methanol released was measured by a gas chromatography (GC) method as previously described and consequently, the DM was calculated [34].

2.3. Modification of pectins

O59 and O64 were re-esterified to obtain high methyl-esterified pectins with a rather random methyl-ester distribution by the use of H_2SO_4 in methanol at

low temperatures (4°C) according to the procedure of Heri et al. [35] to yield $O85_{O59}$ and $O92_{O64}$ respectively.

Random de-esterification of both $O92_{O64}$ and $O85_{O59}$ was done by saponification with diluted NaOH as described previously [36] yielding a set of random methyl-esterified pectins ($O55_{RD64}$ and $O56_{RD59}$) with DM values of 55 and 56%, respectively. The chemical characteristics of the pectin samples are shown in table S1.

2.4. Enzymatic hydrolysis

All citrus pectins were dissolved in 50 mM sodium acetate buffer pH 5.2 (5 mg/ml). Enzymatic hydrolysis was performed at 40 °C by incubation of the pectin solution with PL for 6 h followed by the addition of endo-PG and incubation for another 18 h [37]. Molecular weight distribution was analyzed by High Performance Size Exclusion chromatography (HPSEC). Released diagnostic oligosaccharides were annotated and quantified using High Performance Anion Exchange Chromatography system with Pulsed Amperometric- and UV-detection (HPAEC-PAD/UV) and by Hydrophilic Interaction Liquid Chromatography (HILIC) with online Electrospray Ionization Ion Trap Mass Spectrometry (ESI-IT-MS) HILIC-ESI-IT-MS.

2.5. HPSEC of native and digested pectins

The molecular weight distribution of all (modified) citrus pectins before and after enzymatic digestion was analyzed using a set of four TSK-Gel super AW columns in series: guard column (6 mm ID × 40 mm) and columns 4000, 3000 and 2500 SuperAW (6 mm × 150 mm) (Tosoh Bioscience, Tokyo, Japan) as described previously [30, 38].

2.6. HPAEC of GalA oligosaccharides

The citrus pectin digests were analyzed and subsequently quantified using a HPAEC-PAD-UV system equipped with a CarboPac PA-1 column as described elsewhere [30, 39]. UV detection was used to identify the unsaturated oligosaccharides. GalA DP 1–3 (Sigma Aldrich, Steinheim, Germany) were used as standards for quantification. Oligomers above GalA DP 3 and unsaturated oligomers were quantified using the response of the GalA DP 3 standard.

2.7. HILIC-ESI-IT-MS of methyl-esterified GalA oligosaccharides

Pectin digests were also analyzed using UHPLC in combination with electrospray ionization tandem mass spectrometry (ESI-IT-MS) on a Hydrophilic Interaction Liquid Chromatography (HILIC) BEH amide column. Pectin digests were centrifuged (15000xg, 10 min, RT) and diluted with 50% (v/v) acetonitrile containing 0.1% formic acid, to a final concentration of 1 mg/ml. A heated ESI-IT ionized the separated oligomers in an LTQ Velos Pro Mass Spectrometer (ESI-IT-MS) coupled to an UHPLC and allowed identification of the methyl-esterified oligomers [30].

2.8. Calculating descriptive parameters

Absolute degree of blockiness

The absolute degree of blockiness (DB_{abs}) is calculated as the mole amount of GalA residues present in non-methyl-esterified mono-, di- and trimer released by endo-PG expressed as the percentage of the total moles of GalA residues present in the pectin (Eq. 1) [25, 29].

$$\mathbf{DB_{abs}} = \frac{\sum_{n=1\text{-}3} [\text{saturated GalA}_n\text{released}]_{\text{non-esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (1)$$

Degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme})

To describe the partially methyl-esterified HG region of citrus pectins DB_{PGme} was used [30]. DB_{PGme} is calculated as the number of moles of galacturonic acid residues present in the digest as saturated, methyl-esterified GalA DP 3–8 per 100 moles of the total GalA residues in the pectic polymer (Eq. 2).

$$\mathbf{DB_{PGme}} = \frac{\sum_{n=3\text{-}8} [\text{saturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (2)$$

Degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme})

DB_{PLme} quantifies the amount of unsaturated and methyl-esterified GalA oligomers (DP 2–8) released by the PL. As shown by equation 3, all GalA residues present in unsaturated partly methyl-esterified oligomers (DP 2–8), released by PL action were quantified and expressed as degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}) [30].

$$\mathbf{DB_{PLme}} = \frac{\sum_{n=2\text{-}8} [\text{unsaturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (3)$$

2.9. TLR2/1 inhibiting assays

The HEK- TLR2-1 inhibition assays were performed as described previously [23]. In short, HEK-Blue hTLR2 were pre-incubated with pectins (2 mg/ml). After 1 hour of pre-incubation, cells were stimulated with 10 ng/ml Pam3CSK4 (TLR2-1 agonist), and they were incubated for 24 hours. Culture medium was used as negative control and the Pam3CSK4 was used as positive control. Then, cell supernatant was added to Quantiblue (Invivogen) in a ratio of 1:10. After 1 h of incubation, NF-κB activation was quantified at 650 nm using a Versa Max ELISA plate reader (Molecular devices, Sunnyvale, CA, USA). All incubation steps were performed at 37 °C and 5 % CO_2. The percentage of TLR2-1 inhibition by pectins was calculated by comparing NF-κB activation of pectin-treated cells with the positive control. Each experiment was performed at least five times.

2.10. *In silico* molecular docking

To predict the binding site of pectins to TLR2, docking simulation assays were performed. Two homogalacturonan pectin oligosaccharides of GalA heptamers were chosen as representative compounds. $GalA_7Me^{1,7}$ and $GalA_7Me^{2,5}$ were defined as ligands. GalA residues were annotated 1-7, counting from the reducing end of the oligosaccharide. $GalA_7Me^{1,7}$ and $GalA_7Me^{2,5}$ 3D structures were constructed and edited using the Optical Structure Recognition Software (OSRA) [40]. The crystal structure of the human TLR2-TLR1 heterodimer (PDB code 2Z7X) was used as protein target [41]. This crystallographic structure was obtained in presence of the synthetic bacterial tripalmitoylated lipopeptide Pam3CysSerLys4 (Pam3CSK4) agonist. Thus, the binding agonist pocket could be included as a potential binding site for the chosen pectins. Energy parameters of the ligands and the target were minimized through the Yasara Energy Minimization Server [42].

Molecular docking between TLR2 and pectin oligomers was performed using the protein-small molecule docking web service from the Molecular Modeling Group of the Swiss Institute of Bioinformatics, Lausanne, Switzerland [43]. After docking simulations, the best energy scored poses were selected and considered as the most likely binding structures. Docking simulations, atomic contacts between target and ligands, and their type of interactions were analyzed with Chimera software version 1.14 [44]. Figures were prepared with Pymol Molecular Graphics System version 2.3.5 Edu, Schrödinger, LLC [45].

3. Results and discussion

3.1. Characterization and quantification of pectin diagnostic oligomers

Six pairs of pectins were chosen for their similar DM and their comparable features regarding monosaccharide composition (Table S1) and molecular weight (Mw) distribution (Figure S1). The selected native pectins have been reported before for their bioactivity [12, 46]. In addition, some modified pectins were selected. Two native pectins have been re-esterified and consequently de-esterified close to the DM of the parental pectins. The aim was to discover the bioactivity differences of exactly the same pectins with similar DM, but different DB.

For structural characterization of the pectins, homogalacturonan degrading enzymes endo-PG and PL were used to degrade the pectin backbone and to generate a wide-ranging mixture of diagnostic oligomers. Figure S1 illustrates that all parental pectins had a rather similar Mw. Only chemical modification caused a minor decrease in the Mw of the modified pectins, although all pectins still had a rather similar Mw. HPSEC further shows clearly that endo-PG and PL together sufficiently degraded pectins. The resulting mixture of diagnostic oligomers was then analyzed by HPAEC and HILIC.

HPAEC-PAD/UV of the endo-PG and PL degradation products of pectins allowed the separation, identification, and quantification of GalA monomers and both saturated and unsaturated oligomers ranging from DP 2-7 (Figure 1). The diagnostic oligomer profiles obtained from HPAEC suggest that the pectin pairs all released similar oligomers after degradation. However, as a consequence of pH 12 used during the HPAEC analysis, information on the methyl-esterification of the different oligomers was lost, and therefore, it was not possible to distinguish between methyl-esterified and non-esterified oligosaccharides. To counteract this loss of information on methyl-esters, also HILIC-MS was used to separate and identify methyl-esterified oligomers [30, 47]

and to obtain the relative abundance of selected oligomers after integration of peak areas in the ion chromatograms as described previously [30]. By combining HPAEC and HILIC-MS data, the methyl-ester distribution patterns of pectins were characterized in detail (Figure 2).

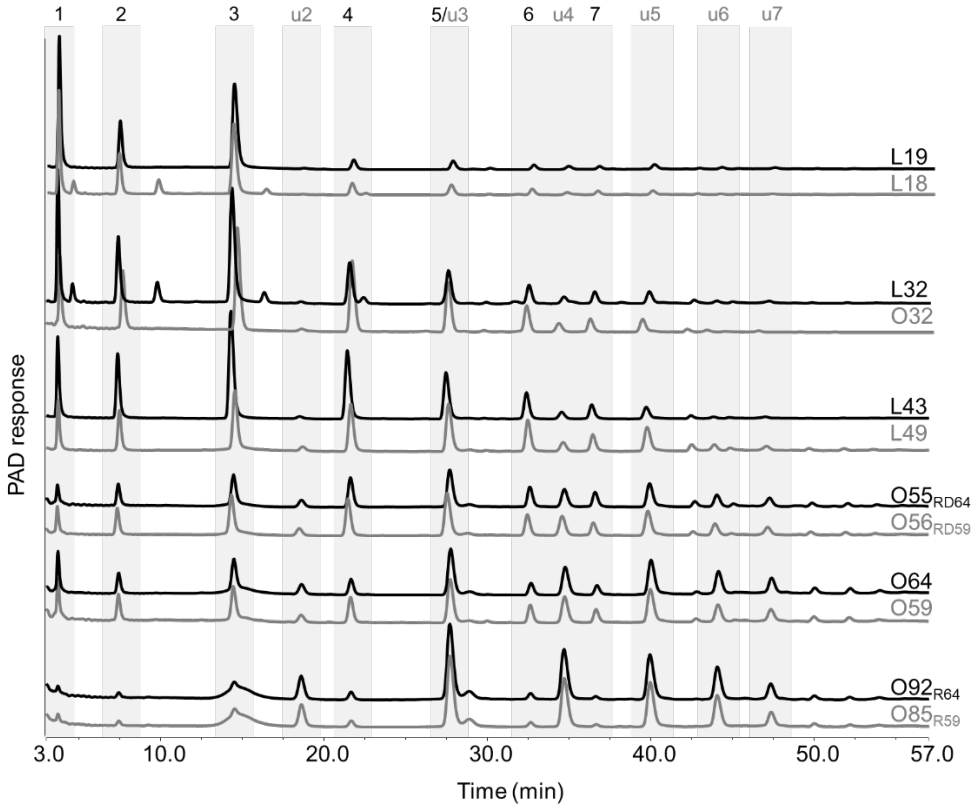

Figure 1. *HPAEC-PAD elution patterns of endo-PG and PL digests of pectins after 24h incubation detected by PAD. Peak annotation: 4, saturated DP4 GalA oligosaccharide; u4, unsaturated DP4 GalA oligosaccharide. Pectin codes: O: orange origin, L: lemon origin, Number: DM. L18 = Lemon pectin with a DM of 18, RD: pectin has been re-esterified and consequently de-esterified using alkali from parental pectin, R: pectin has been re-esterified from parental pectin*

The diagnostic oligomers as present in different ratios in the HILIC elution patterns of the PG-PL enzyme digests of the twelve citrus pectins, indicated diverse methyl-ester distribution patterns for the rather similar DM pectins (Figure 2). Besides the non-esterified GalA 2^0 and 3^0, it can be clearly seen that both saturated and unsaturated oligomers having the same DP and different levels of methyl-esterification such as 4^1, 4^2, $u4^2$, $u4^3$ etc, were also nicely separated. However, a complete chromatographic separation of all

GalA isomers, i.e., oligomers merely varying in the position of methyl-esters was not attained, but distinction could be obtained by extracted ion chromatograms [48].

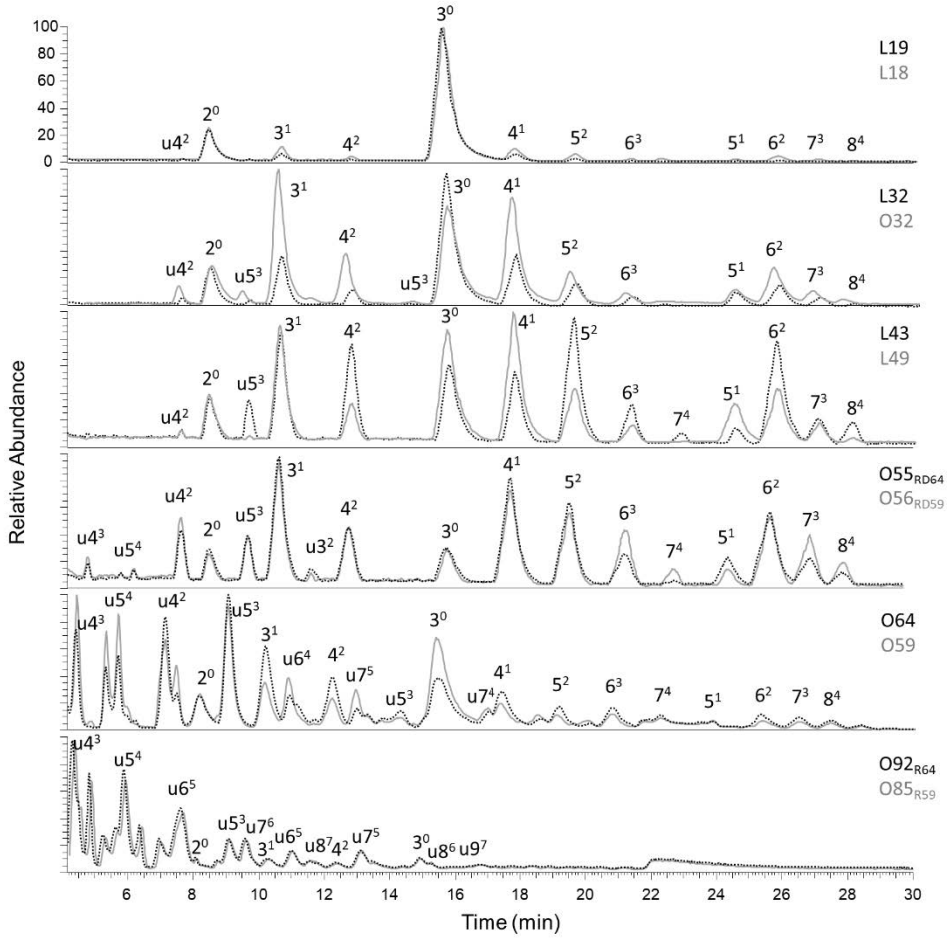

Figure 2. *HILIC-MS base peak elution pattern of pectins digested by the enzymes endo-PG and PL. Peak annotation: 3^1, saturated DP3 GalA oligosaccharide having one methyl-ester; $u5^3$, unsaturated DP5 GalA oligosaccharide having three methyl-esters Pectin codes: O: orange origin, L: lemon origin, Number: DM. L18 = Lemon pectin with a DM of 18, RD: re-esterified and consequently alkali de-esterified pectin, R: re-esterified from parental pectin.*

To visualize the differences in the oligomer profiles of pectins, especially for the similar DM pectins, a bar chart has been created. Figure 3 clearly shows how much the released oligomers differ in amount for digests from e.g., the pectin pairs. The figure visualizes the relative amounts of the various diagnostic oligomers, as released by PG (saturated, non-esterified mono-, di-

and triGalA and methyl-esterified oligosaccharides) and the unsaturated, methyl-esterified oligomers released by PL. As expected, the level of oligomers released by PG decreased with an increase in DM and, at the same time, the amounts of oligomers released by PL were increasing.

The figure is quite revealing in several ways. First, a rather big difference has been observed between pectins L18 and L19, regardless of the 80% non-esterified GalA residues in the backbone. As expected DP1-3 were the most dominant products but differed slightly in amount. Looking at the yellow and blue segments (Figure 3), it can be seen that L18 had more methyl-esterified oligomers released by PG, than L19, and the PL degradation products also varied for the two pectins. The PL degradable regions of L32, O32 and even L43 pectins were similarly minor as the very low DM pectins L18/19 PL degradable regions. In the aforementioned pectins, the level of PG degradable completely non-esterified and partially esterified regions however, shifted compared to the L18/19 pectins as expected. Looking at the degradation profiles of the two parental high DM pectins O64 and O59 and the modified $O55_{RD64}$ and $O56_{RD59}$ pectins, it can be seen that while the parental pectins had quite different degradation products, after the modification, their profiles became fairly similar. Furthermore, the re-esterified $O92_{R64}$ and $O85_{R59}$ pectins similarly to the very low DM pectins still showed different degradation products upon digestion and as expected primarily unsaturated PL oligomers dominated.

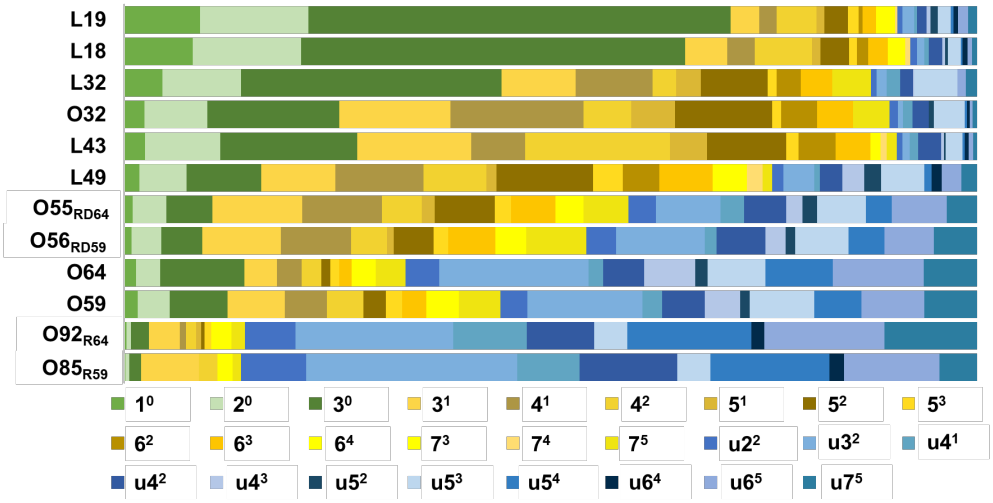

Figure 3. *Relative abundance of released diagnostic oligomers of citrus pectins after incubation with endo-PG and PL. Oligosaccharides were quantified using HPAEC-PAD and HILIC-MS. Annotation: u3[2] , u = unsaturated, 3 = number of galacturonic acid residues, superscript [2] = number of methyl-esters present on the GalA residue. L: lemon origin, O: orange origin, Number: DM. L18 = Lemon pectin with a DM of 18, R: re-esterified pectin, RD: re-esterified and consequently alkali de-esterified pectin, green colours represent non-Me GalA oligomers released by PG; yellow colours represent Me GalA oligomers released by PG; and blue colours represent unsaturated Me GalA oligomers released by PL.*

From Figure 3, it is apparent that pectins having similar DM values show noticeably different patterns. Prior studies have already noted the importance of characterization of methyl-esterification patterns in pectins [25, 29, 49]. Using the simultaneous endo-PG and PL digestion and combined HPAEC and HILIC analysis to separate and quantify pectic oligomers released from these citrus pectins helped to realize that similar DM pectins can have different methyl-ester distribution.

3.2. Descriptive parameters of pectin

Parameters highlighting structural features of pectin's methyl-esterification

The differences in methyl-ester distribution patterns of citrus pectins can be described by the parameters DB_{abs}, DB_{PGme} and DB_{PLme} [25, 30]. These parameters were calculated from the amounts of specific oligosaccharides released from the various pectins used in this study. As it was apparent already from Figure 3 that the quantification of diagnostic oligosaccharides resulted in quite

different descriptive parameters. Consequently, these parameters allowed us to identify different methyl-ester distribution patterns of pectin pairs, regardless of their similar DM [30].Figure 4 illustrates in a simplified way that two high DM pectins can have considerable variations especially in the methyl-esterified sections of the backbone. Depending on the position of the methyl-esters, PG and PL will cut the backbone at different positions resulting in different diagnostic oligomers.

A high DB_{abs} indicates a more blockwise distribution of non-esterified GalA residues in the pectin. The methyl-esterified diagnostic oligomers liberated by PG represented by the DB_{PGme} are the less methyl-esterified segments of pectin which still have a pattern of methyl-esterification outside the non-esterified blocks. In addition, DB_{PLme} represents the highly methyl-esterified oligomers released by PL. In DB_{PLme} oligomers the methyl-esters are more closely associated than in the DB_{PGme} oligosaccharides. The differences in DB_{PGme} and DB_{PLme} already suggested more refined structural differences in the pectin pairs. Moreover, the ratio of DB_{PGme}/DB_{abs} has also been introduced, which is the ratio of moderately methyl-esterified GalA oligomers (DB_{PGme}) and the completely non-esterified GalA oligomers (DB_{abs}), both of which are released by PG. The DB_{PGme}/DB_{abs} ratio indicates a distinct distribution pattern of the non-esterified GalA blocks over the backbone.

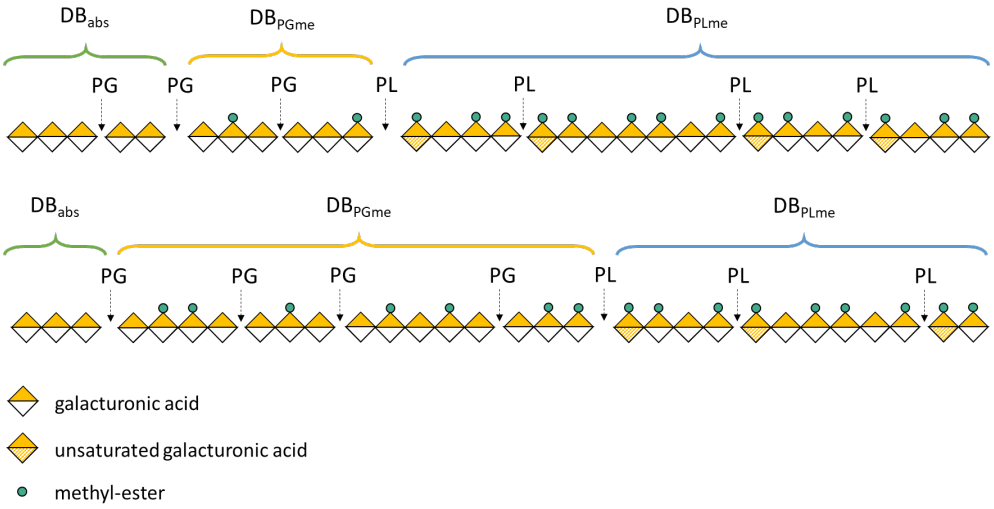

Figure 4. *Schematic representation of a hypothetic backbone of two high DM pectins with different methyl-ester distributions after combined digestion of PG and PL including the descriptive parameters DB_{abs}, DB_{PGme} and DB_{PLme}. The sequence of oligosaccharides is hypothetical.*

Methyl-esterification patterns in pectins studied

Table 1 shows the descriptive parameters for the six pectin pairs used in this study. The pectin pairs differ in the distribution of methyl-esters over the pectin's backbone as illustrated by their DB_{abs}, DB_{PLme} and DB_{PGme}. In general, for all six pectin pairs, the DB_{PGme}/DB_{abs} ratio was lower for the higher DB_{abs} pectins of the similar DM pectin pairs.

Table 1. *Descriptive parameters and TLR 1/2 inhibition of commercial and modified pectins used in this study*

Sample[a]	DM[b]	DB_{abs}[c]	DB_{PLme}[d]	DB_{PGme}[e]	DB_{PGme}/DB_{abs}	TLR 2/1 [f]inhibition (%)
L19	19	75	10	21	0.3	54
L18	18	66	8	27	0.4	48
L32	32	48	14	47	1.0	51
O32	32	24	10	60	2.5	35
L43	43	32	11	73	2.3	62
L49	49	18	26	65	3.7	45
$O55_{RD64}$	55	10	38	46	4.7	24
$O56_{RD59}$	56	8	39	38	4.9	23
O64	64	14	65	18	1.3	45
O59	59	12	53	30	2.6	28
$O92_{R64}$	92	3	75	10	3.9	30
$O85_{R59}$	85	2	99	14	5.9	17

[a] O: orange origin, L: lemon origin, Number: DM. L18 = Lemon pectin with a DM of 18, RD: pectin has been re-esterified and consequently de-esterified using alkali from source pectin, R: pectin has been re-esterified from source pectin
[b] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample.
c Absolute degree of blockiness (DB_{abs}): the amount of non-esterified mono-, di- and triGalA per 100 mol of total GalA in the sample.
[d] Degree of blockiness by PL (DB_{PLme}): the amount of methyl-esterified unsaturated galacturonic oligomers per 100 mol of total galacturonic acid in the sample.
[e] Degree of blockiness by endo-PG (DB_{PGme}): the amount of saturated methyl-esterified galacturonic residues per 100 mol of total galacturonic acid in the sample.

DM ~20 pectins

For the very low DM pectins the DB_{abs} was the highest and DB_{PLme} was the lowest of all pectins, just as expected, as 80% of the backbone is non-esterified and the methyl-esters can not be too closely positionated. Compared to pectin L19, it can be seen that the DB_{abs} and DB_{PLme} for pectin L18 was somewhat lower while the DB_{PGme} was higher, which points out that the methyl-ester distribution differed for the two pectins even though both of them had a very low DM. Looking at the DB_{PGme}/DB_{abs} ratio for L19, it was lower than for L18, but still rather similar (0.3 and 0.4 respectively).

DM ~30 pectins

Between the low DM30 pectins, there were considerably higher differences. L32 and O32 had highly different DB_{abs} and DB_{PGme} values, while their DB_{PLme} values were somewhat similar. The DB_{abs} of O32 pectins was found to be half of L32 (24 and 48 respectively) which suggests a very random distribution of the O32 pectin. The high DB_{PGme} of the O32 pectin supports the low DB_{abs} value, referring to parts of the backbone which are methyl-esterified in such a way that PG can still act. The DB_{PLme} of the DM30 pectin pair was fairly similar meaning that also more densely methyl-esterified segments of the backbone were present and in rather comparable amounts. It can thus be suggested that the PL degradable methyl-esterified segments of both of the pectins were fairly similar, while the PG degradable non-esterified segments in L32 pectin were rather long, and in O32 they were interrupted with methyl-esters. The ratio of DB_{PGme}/DB_{abs} was also 2.5 times higher for the O32 pectin, suggesting a random distribution of methyl-esters.

DM ~45 pectins

For the intermediate DM pectins L43 and L49, a fairly different trend was shown since their DB_{abs} and DB_{PLme} values were greatly different while their DB_{PGme} were comparable also to the DM~30 pectins. Suggested by the higher DB_{abs} L43 had longer blocks of non-esterified GalA residues compared to L49. Interestingly the high DB_{PGme} and low DB_{PLme} values propose that the methyl-esterified segments were actually more randomly distributed over the backbone for L43, despite having a higher $DB_{abs.}$ L49 had less blockwise non-esterified GalA distribution. The higher DB_{PLme} for L49 showed that the methyl-esters over the backbone were more closely associated compared to the L43 pectin. This means that L49 pectin had a random distribution in the PG degradable segments, while in the PL degradable segments the methyl-esters were distributed closer together.

DM ~60 pectins

O64 and O59 had comparable DM and DB_{abs} values (14 and 12 respectively). DB_{PLme} was higher for O64 compared to O59 and DB_{PGme} of O64 was almost half of O59. It is believed that O64 pectin, while having somewhat longer non-esterified blocks, also had closely associated methyl-esters distributed over the backbone, compared to the more random O59. The ratio of DB_{PGme}/DB_{abs} was also much lower in O64 pectin compared to O59 (1.3 and 2.6 respectively), further supporting the different methyl-ester distributions. The structural differences between the two commercial pectins were striking, as they were produced by the same company, extracted from the same raw material and had similar DM.

Re-esterified, DM ~90 pectins

The very high DM pectins $O85_{R59}$ and $O92_{R64}$ have fairly low chances of having blocks of non-esterified GalA sequences, which is also shown by their rather low DB_{abs} (2 and 3 respectively). The very high DM is recognised as well by the very high values for DB_{PLme} (75 and 99 respectively). Yet surprisingly $O85_{R59}$ and $O92_{R64}$ pectins still had their own, slightly different, methyl ester distributions over their backbone as shown by their DB_{PGme} (14 and 10 respectively) and the ratio of DB_{PGme}/DB_{abs} (5.9 and 3.9 respectively). The aim of re-esterification was to create two similar, fully esterified pectins, however, they both kept some of the properties of their parental pectins. Unlike what has been suggested by Daas et al. [28] and others, re-esterification of pectins to DM>90 is not sufficient to obtain a fully randomly methyl-esterified pectin.

De-esterified, DM ~55 pectins

The re-esterification and consequent de-esterification of the blocky O64 pectin resulted in a highly random pectin $O55_{RD64}$, which can be seen also from the lower DB_{abs} and a substantial increase in DB_{PGme}/DB_{abs} ratio compared to the parental pectin. The DB_{PGme}/DB_{abs} ratio was the highest for the two randomized pectins, $O55_{RD64}$ and $O56_{RD59}$ (4.7 and 4.9 respectively). In general, the two de-esterified pectins became fully random compared to the parental pectins but $O55_{RD64}$ was found to be more blockwisely distributed, just as its parental O64 pectin.

The data indicated by the DB_{PGme}/DB_{abs} ratio obtained after combined PG and PL digestion of pectins can be probably best explained by the parental and modified pectins. As expected, the DB_{PGme}/DB_{abs} ratio was the lowest for

pectins releasing higher amounts of non-esterified GalAs and lower methyl-esterified oligomers. For the randomized pectins $O55_{RD64}$ and $O56_{RD59}$ the value of DB_{PGme} increases and DB_{PLme} decreases compared to the parental pectins, which indicates a random pattern of methyl-ester distribution. This suggests that the arrangement of the methyl-esters over the backbone allows more PG action and the release of saturated non-esterified mono-, di- and tri-GalA and also various methyl-esterified oligomers, and decreases the chances of PL to act as the methyl-esters are less closely associated on the homogalacturonan. As a result, a randomly methyl-esterified pectin would have an increased ratio of DB_{PGme}/DB_{abs}. Surprisingly the two randomized pectins did not become equally distributed, despite the same treatment and similar DM.

3.3. Methyl-ester distribution patterns of citrus pectins drive TLR2/1 inhibition

It has been found that citrus pectins can influence immunity through Toll-like receptor (TLR) signalling [10, 12]. TLR2-TLR1 dimerization is specifically activated by a Pam3CSK4 agonist and the dimerization will induce proinflammatory pathways, therefore inhibiting the TLR2/1 dimerization using pectins can potentially prevent inflammation [11, 46, 50]. The inhibition of TLR2/1 was studied by using the Pam3CSK4 agonist. The TLR1/2 inhibiting capacities of the set of pectins can be seen in Table 1.

Low DM pectins L18, L19 having both low and high DB values all strongly inhibited TLR2/1. L32 pectin with a high DB has shown just as strong inhibition as the L18/19 pectins, while O32 with a low DB inhibited TLR2/1 31% less than the same DM L32 pectin with a high DB. Intermediate DM pectin L49 with a low DB inhibits similarly to the low DM pectins, and surprisingly L43 with a high DB inhibit about 20% stronger than the low DM pectins. Among the high DM pectins, O64 having a high DB has shown the strongest inhibition, while the other high DM pectins did not inhibit TLR2/1 as strongly.

Previously it was shown that the impact of citrus pectins on TLRs depends on the DM [10, 11]. A strong relationship between the methyl-ester distribution parameter DB and the TLR2/1 inhibition has been reported by Beukema et al. [12], suggesting that methyl-ester distribution patterns of pectins play a role in TLR2/1 binding. The presence of distinct blocks of non-methyl-esterification is more important for a good binding and inhibition than the overall charge of the pectin as determined by the DM. However, both DM and DB could not fully explain the inhibition for all pectins as published before [12, 46]. Therefore more pectins were chosen in this study, including methyl-ester-distribution modified pectins and the TLR2/1 inhibition were measured for all pectins.

In search of the descriptive parameter that would explain the level of TLR2/1 inhibition, it was found that the ratio of DB_{PGme} to DB_{abs} showed the highest correlation to the TLR2/1 inhibition. DB_{PLme} has been shown not to contribute to the correlation (results not shown). It is striking from Figure 5, that for example, O64 pectin with a (low) DB_{PGme}/DB_{abs} ratio of 1.3 inhibits TLR2/1 stronger than lower DM pectins and higher DB pectins. Since the DB_{abs} is not correlating similarly as the DB_{Pgme}/DB_{abs} ratio, it is clear that not only a long stretch of non-methyl-esterified GalA residues is necessary for optimal binding.

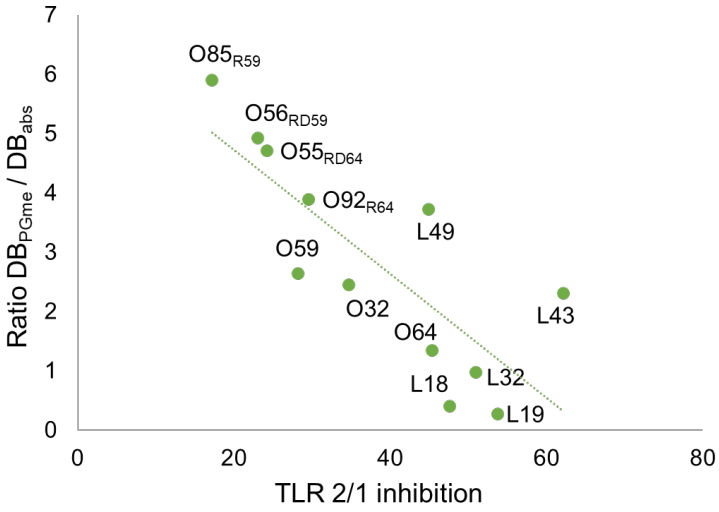

Figure 5. *Ratio of TLR2/1 inhibition plotted versus the DB_{PGme} : DB_{abs} of pectin digests. R^2=0.64. Negative correlation is shown between the TLR2/1 inhibition and the DB_{PGme} : DB_{abs} ratio. DB_{PGme} : DB_{abs} is the ratio of all methyl-esterified saturated oligos to the non-esterified saturated oligos degraded by PG. Inhibition of TLR2/1 by citrus pectins. HEK-Blue™ hTLR cells were first pre-incubated for 1 h with pectins (2 mg/ml) and subsequently stimulated with the Pam3CSK4 agonist.*

The L18 and L19 pectins both belong to the most strongly TLR2/1 inhibiting pectins, as already claimed before for LM pectins [11]. Our hypothesis that there is a certain pattern of methyl-esterification needed for TLR2 binding is underpinned by the finding that DM0 pectin (polygalacturonic acid) bound to TLR2 less than low DM pectins [50]. Our results suggest that most probably, next to a non-esterified GalA segment, also a PG degradable segment with a specific methyl-ester distribution is important for binding to TLR2. O32 and L49 were found to inhibit TLR2/1 less than L32 and L43, which corroborates the findings that the TLR2 binding cannot be exclusively explained by the

DM or DB. It is also important to note that DB_{abs} does not offer information on the size of non-esterified blocks [25, 51]. The non-esterified block sequence in pectins with a remarkably high DM, such as $O92_{R64}$ and $O85_{R59}$ and in the successfully randomized $O55_{RD64}$ and $O56_{RD59}$ pectins, is probably too short to induce TLR2/1 inhibition. The patterns as indicated by the DB_{PGme}/DB_{abs} ratio in high and intermediate pectins such as O64, L43 and the low DM pectins L19, L18 and L32 pectins are highly inhibitory for TLR2-TLR1 dimerization. An explanation for these differences might be that the combination of non-esterified block size and distribution of methyl-esters both play a role in the TLR2/1 inhibition by pectins as also indicated by the DB_{PGme}/DB_{abs} ratio.

These results provide further support for the hypothesis that pectin inhibits TLR2/1 dimerization by binding to amino acids on the TLR2 binding sites by presumed electrostatic interactions [11, 50]. High DB_{abs} pectins have many negatively charged GalA in sequence, which can possibly interact with the TLR2 ectodomain [52]. Even though the number of non-methyl-esterified GalA and consequently the non-esterified blocks in low DM pectins is certainly more than in high DM pectins, there is a given pattern of methyl-esterified GalAs needed for the inhibitory effect.

3.4. Pectins interact with different TLR2 sites in a pattern dependent fashion

In our study, pectins with a certain block size of non-esterified GalA residues next to sequences of methyl-esterified GalA residues had a stronger inhibitory effect on TLR2. The molecular docking analysis was performed to gain insight into the molecular mechanisms that drive this inhibitory effect of pectins on TLR2 and to validate our hypothesis that a specific distribution or pattern of methyl-esters plays an important role. To foresee whether there is a specific methyl-ester distribution pattern over the GalA backbone of pectins that binds stronger to TLR2, two heptamers of GalA residues that differed in methyl-ester distribution were modelled for their best fit to interact with the human TLR2 (PDB code 2Z7X). One heptamer contained methyl-esters at GalA residues #1 and #7 (counting from the reducing end) ($GalA_7Me^{1,7}$), which leads to a sequence of 5 non-esterified GalA residues. The other heptamer contained methyl-esterified GalA residues at positions #2 and #5 ($GalA_7Me^{2,5}$), representing two shorter segments of 2 non-esterified GalA residues (Figure S3).

The best-ranked pose of $GalA_7Me^{1,7}$ had a binding energy to TLR2 of -10.94 kcal/mol, which was located at the heterodimer TLR2/1 interface (Figure 6A-B). Key amino acid residues from TLR2 which participate in the TLR2/1 interface made contact with $GalA_7Me^{1,7}$: amino acid residues E369 N345 and

H398 interacted through hydrogen bonds, and K347 made contact by electrostatic interactions (Figure 6C). The O-methyl group at GalA #1 was found to interact with Glu residue #369, while methyl substitution at GalA #7 did not make any contact with TLR2 (Figure 6C).

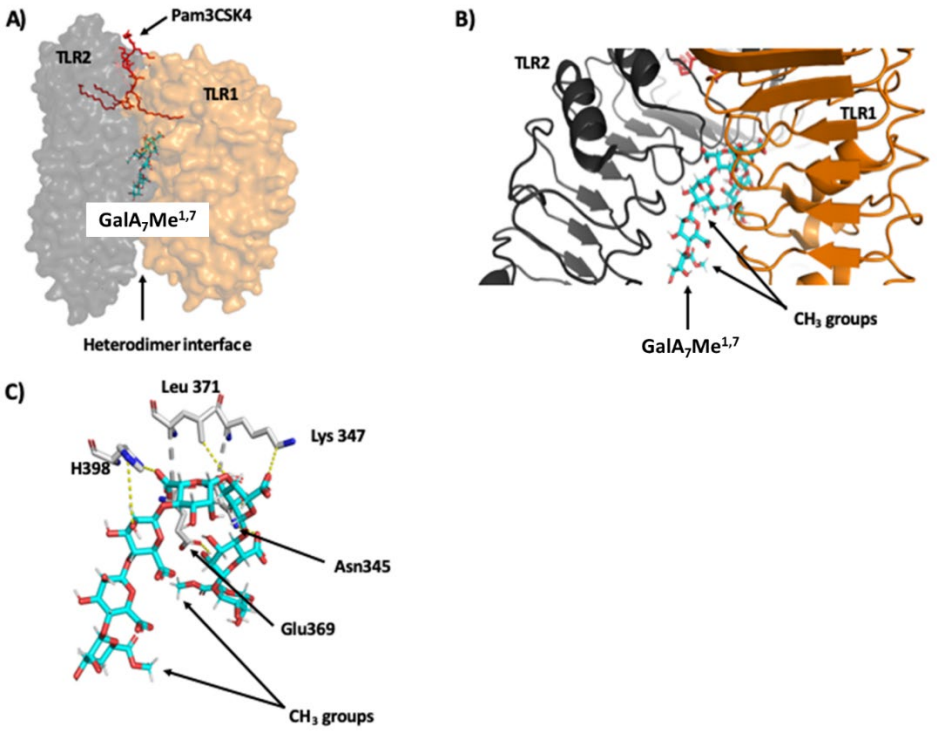

Figure 6. *Binding mode prediction of $GalA_7Me^{1,7}$ to hTLR2-TLR1 heterodimer by docking simulation.* ***A)****. Overview of hTLR2-TLR1 heterodimer and $GalA_7Me^{1,7}$ interaction. The target protein is represented in surface. The pectin ligand is represented as sticks. TLR2 agonist Pam3CSK4 is depicted in red.* ***B)****. Close up to the $GalA_7Me^{1,7}$ predicted interaction site, the protein is represented in cartoon. C). Interface TLR2 amino acid residues interacting with $GalA_7Me^{1,7}$ are represented in sticks, yellow dotted lines indicate atomic contacts.*

For $GalA_7Me^{2,5}$, the best-ranked pose had a less favorable binding energy value of -3.43 kcal/mol. $GalA_7Me^{2,5}$ was found on TLR2 central domain (Figure 7A), contacting amino acid residues of the leucine-reach repeats (LRRs) 7-10 through hydrogen bonds (Figure 7B). None of the two methyl groups from $GalA_7Me^{2,5}$ interacted with TLR2 (Figure 7B). Together these results show that the heptamer representing a longer non-esterified block ($GalA_7Me^{1,7}$), is more efficient in binding to TLR2 interface than the pectin heptamer representing a block of only 3 non-esterified GalA residues ($GalA_7Me^{2,5}$), which may be

explain the strong TLR2/1 inhibiting properties of pectins with higher degree of blockiness.

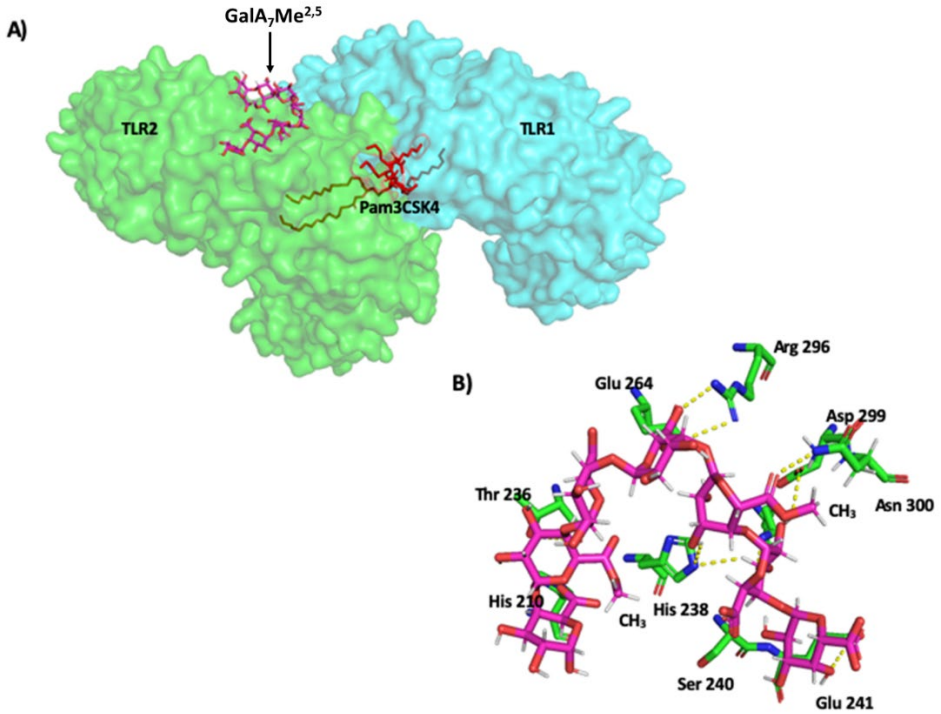

***Figure 7**. Docking simulation for interaction site prediction of $GalA_7Me^{2,5}$ with hTLR2-TLR1 heterodimer. Target protein and ligand are represented in surface and sticks, respectively. **A)** Top overview of the predicted binding mode of $GalA_7Me^{2,5}$ to hTLR2. **B)** Detailed interaction of $GalA_7Me^{2,5}$ with amino acid residues of the TLR2 central domain, dotted lines indicate atomic contacts.*

Our docking study demonstrates that a block of non-esterified GalA sequence can bind to TLR2 at the heterodimer interface. This refines our previous finding about the capacity of pectin to bind TLR2 [11]. It is known that the activation and further signaling of TLR2/1 is induced by the binding of the agonist at the central domain of the complex. The agonist binding plays a key role in the approximation of TLR2 and TLR1 and the consequent formation of the TLR2/1 heterodimer-agonist complex. When TLR2 and TLR1 get sufficiently close to each other by the binding of the agonist, other amino acid residues located below the agonist-binding site participate in the formation of this TLR2/1 interface further stabilizing the complex [41]. Herein we demonstrate in more detail that this binding of pectin at the TLR2/1 interface site prevents the stabilization of the TLR2/1 complex, which could

explain the inhibitory effect observed. Previously it has been shown that inhibition of TLR2 by food components can attenuate inflammatory responses [53].

Pectic oligosaccharides vs polysaccharides

Based on the docking studies using oligomers and the TLR2/1 inhibition of the twelve polymeric pectins, it can be concluded that pectin conformation also plays a role in the binding to the TLR2. Depending on the pattern of methyl-esterification, the intramolecular and intermolecular interactions and three-dimensional conformation of pectins in solution will vary [28, 54]. Pectin can form a gel when calcium is present and for that a block of at least 8-12 consecutive non-esterified GalA residues is needed [24].

L19, L18, L32, L43, L49 and O64 are the most capable pectins to prevent binding of the TLR2 ligands and by that, inhibit TLR2/1 dimerization. At least 5 non-esterified GalA residues need to be free to be able to bind to TLR2, although efficient binding of the segment strongly depends on the three-dimensional conformation of the entire pectin polymer and the number of such binding sites present. Vogt, Sahasrabudhe [10] have shown that pectic oligomers did not activate TLRs. When TLR2 has a pectin polymer bound to it, the size of the polymer may prevent the binding of the agonist even to a different binding site and by that inhibiting the dimerization with TLR1 [12].

Not only the blockwise distribution of non-esterified GalA residues is important for TLR2/1 inhibition which can be confirmed by the finding that low DM, intermediate DM, and even high DM pectin with a relatively low ratio of DB_{PGme}/DB_{abs} inhibited TLR2/1 dimerization. This finding suggests that a certain non-esterified block size between (partially methyl-esterified GalA residues is important for the ability of pectins to bind to TLR2 and with that to prevent TLR1 to dimerize. The modelling clearly demonstrated that a sequence of 5 non-esterified GalA is more potent for inhibition than a sequence of 3 non-esterified GalA residues for binding to TLR2. More or too many suitable patches within a large pectin molecule might not increase the inhibition due to steric hindrance. The optimal stretches of non-esterified GalAs, and the methyl-esterification patterns together make the non-esterified blocks not too long, but also not too short. The outcomes of the docking analysis are highly valuable, although these findings are somewhat limited by the fact that only two heptamers of GalA residues were used for the docking analysis. More modelling would be needed to reveal more insights on the binding of the homogalacturonan to the TLR2.

4. Conclusion

The main goal of the current study was to determine the structure-function relationship between pectins and TLR2/1 inhibition. Pectins were characterized by enzymatic fingerprinting methods and their structural characteristics were studied to understand their interaction with TLR2/1 and to explain observed inhibition of TLR2/1 by specific pectins.

Our methodology of enzymatic fingerprinting to characterize pectins and descriptive parameters DB_{PGme} and DB_{PLme} have been demonstrated to be extremely powerful to differentiate between major and minor differences in the methyl-ester distribution of pectins [30]. It also has been shown that pectins with rather equal DM and even equal DB_{abs} values are quite different in structure and also their behavior is different. Depending on the application, such small differences can be relevant.

Since it is difficult to understand the behavior of almost similar pectins in TLR binding, molecular docking analysis was used to visualize the binding mechanism to TLR2. The results may explain the mechanisms by which pectins are able to have immunomodulating properties [11, 12]. As TLR2/1 inhibiting pectins have been shown to limit the development of mucositis and other intestinal disorders [46]. Our data suggest that pectins can have beneficial effects on human health. Although more detailed studies are needed, our findings certainly add to the understanding of the beneficial immunomodulatory effects of pectins, which may be explained by their impact on TLR2 and decrease of proinflammatory responses. The findings reported here shed new light on the fact that the methyl-esterification pattern of a citrus pectin is a highly valuable structural and functional feature and can determine the TLR2 binding capacity of the pectin.

Our findings clearly illustrate that the structure of pectins should be profoundly characterized. Further research is needed to establish which pectins specifically serve best as immune supporting molecules. As TLR2 signaling is involved in intestinal disorders this data suggests that pectins potentially have beneficial effects on inflammatory disorders.

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Appendix

Table S1. *Characteristics of citrus pectin samples used in this study*

Pectin [a]	Rha	Ara	Gal	Glc	GalA[b]	Total[c]	Mw[d]
			(mol%)			(w/w%)	(kDa)
L19	1.2	1.4	14.1	1.1	81	65	75
L18	0.9	0.6	9.9	0.8	88	63	78
L32	0.9	0.5	7.2	0.5	91	69	70
O32	1.0	3.0	6.0	1.0	89	87	77
L43	0.7	0.3	2.7	0.5	96	64	79
L49	0.8	3.4	6.2	0.6	89	70	114
O64	0.0	7.0	7.0	1.0	84	86	92
O59	1.0	3.0	9.0	3.0	84	83	87
O92$_{R64}$	0.0	0.0	7.0	1.0	91	74	62
O85$_{R59}$	1.0	0.0	8.0	2.0	89	84	55
O55$_{RD64}$	1.0	0.0	8.0	2.0	89	73	60
O56$_{RD59}$	0.0	0.0	9.0	3.0	88	71	54

[a] L: lemon origin; O: orange origin; Number: DM; L19 = Lemon pectin with a DM of 19, RD: pectin has been re-esterified and consequently de-esterified using alkali from source pectin, R: pectin has been re-esterified from source pectin
[b] Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, GalA = Galacturonic acid.
[c] Total sugar content in w/w%
[d] Molecular weight (Mw) in kDa as measured by HPSEC

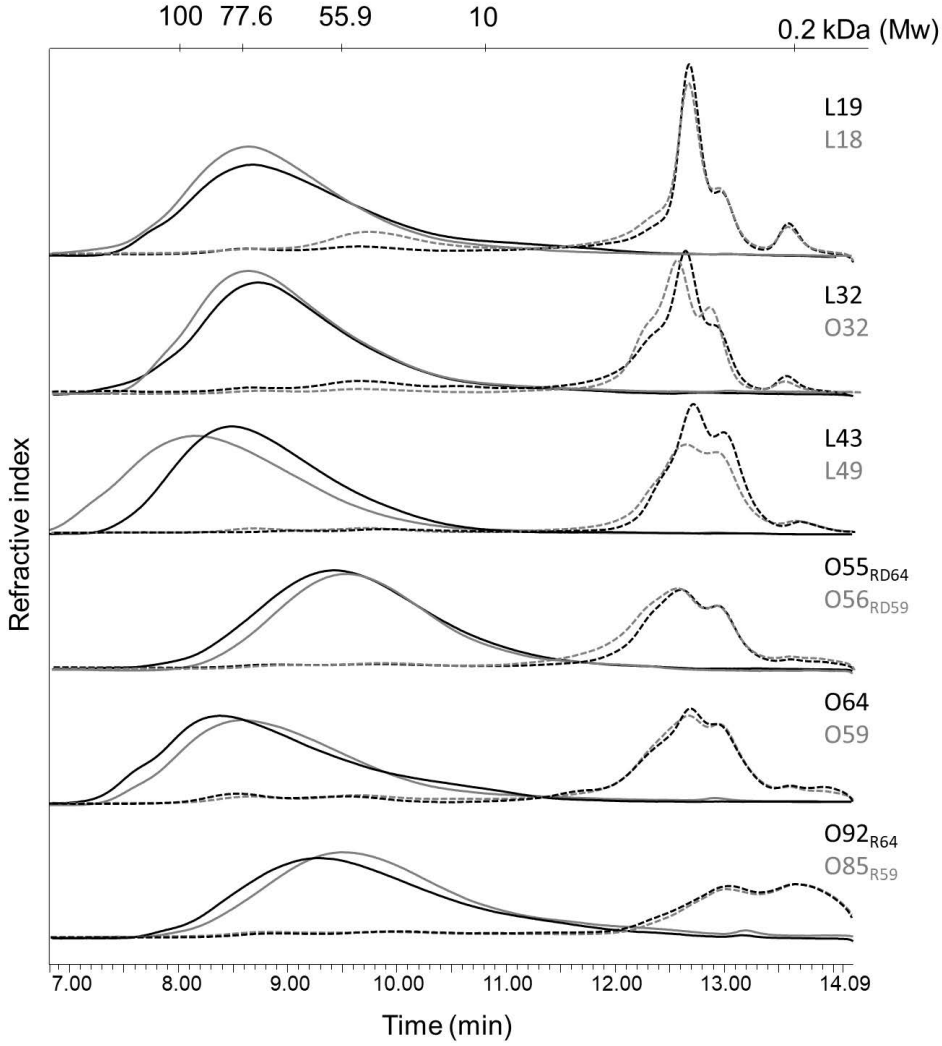


Figure S1. *HPSEC elution profiles of pectins before (solid line) and after (dashed line) digestion by homogalacturonan degrading enzymes: PL and endo-PG. Molecular weights of pectin standards (in kDa) are indicated.*

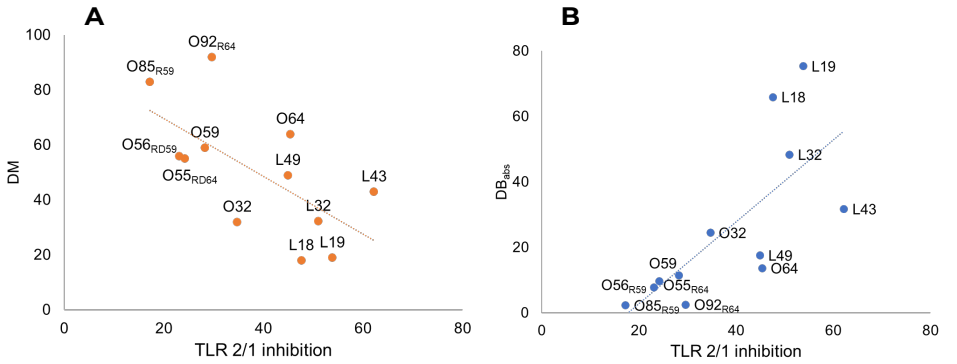


Figure S2. A) *Ratio of TLR2/1 inhibition plotted versus the DB_{abs} of pectin digests. R^2=0.42* ***B)*** *Ratio of TLR2/1 inhibition plotted versus the DM of pectin digests. R^2=0.52.*

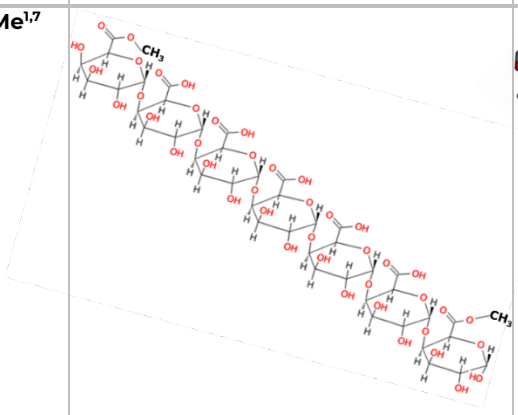
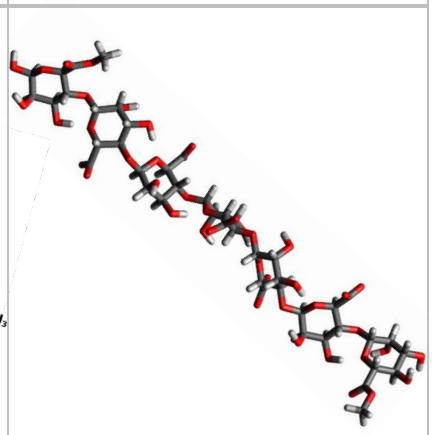
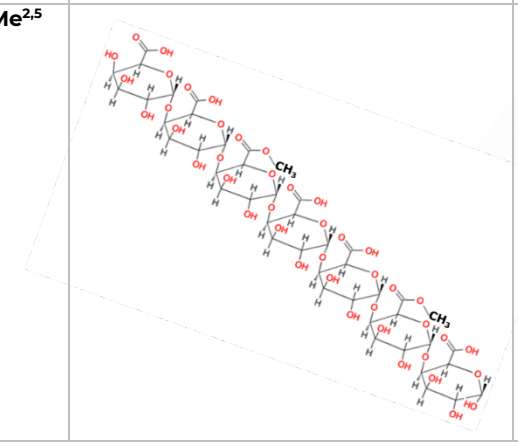
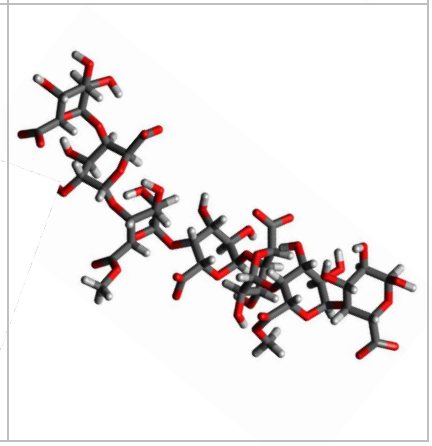
Pectin	2D structure	3D structure
$GalA_7Me^{1,7}$		
$GalA_7Me^{2,5}$		

Figure S3. *2D and 3D structures of $GalA_7Me^{1,7}$ and $GalA_7Me^{2,5}$ GalA heptamers used for the docking experiments. Oligomers are drawn with their reducing end to the right-side of the heptamer*

Chapter 5

Structure-dependent effects of low doses of pectins on the gut microbiota composition and fermentation in different murine intestinal segments

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submitted for publication

Abstract

This study aimed to characterize the structure-function relationship between pectins that differ in degree of methyl-esterification (DM) and degree of blockiness (DB) in different parts of the mouse intestine. Modulation of composition and activity of murine intestinal-microbiota, short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFS) production and Aryl-hydrocarbon-receptor (AhR) activation was quantified. Healthy mice were supplemented with structurally different lemon pectins (3 mg/day) for 1 or 4 weeks. It was found that pectins, DM19 with a high degree of blockiness (HB), DM43 (HB) and DM49 with a low degree of blockiness (LB) effectively increased microbiota diversity, especially in the large intestine. In the caecum, these three pectins induced a higher relative abundance of Lachnospiraceae*, Alloprevotella, Prevotellaceae,* and *Akkermansia* and activation of AhRs were found. The main properties of pectins that are linked to shifts in gut microbiota composition are DM and DB. This study indicates that 0.1% addition of pectins in feed can stimulate beneficial intestinal bacteria, and promote immune activities through AhRs in a structure-dependent way.

1. Introduction

Pectins are complex polysaccharides having a galacturonic acid (GalA)-rich backbone with GalA residues being organized in a linear or "smooth" homogalacturonan (HG) and a branched or "hairy" rhamnogalacturonan I (RG I) region [1, 2]. The HG backbone consists of linear α-(1,4)-linked D-galacturonic acid units, which are partially methyl-esterified at the C-6 carboxyl group and, less frequently acetylated at the O-2 and/or O-3 position [3]. The percentage of the methyl-esterified GalA residues over all GalA residues present within the HG backbone is defined as the degree of methyl-esterification (DM). The distribution of methyl-esters over the HG is described by the degree of blockiness (DB) [4]. DB represents the amount of non-esterified mono-, di- and trigalacturonic acids released by enzymatic treatment of pectin using endo-polygalacturonase (endo-PG) relative to the total amount of non-esterified GalA residues present in the pectin [4]. While DB_{abs} is related to the total amount of GalA residues present in the pectin, DB and DB_{abs} are commonly used to differentiate methyl-esterification patterns of pectins [5]. High DB pectins have a more blockwise distribution of non-esterified GalA over the HG backbone than low DB pectins, which show a rather random distribution [4, 5]. Interestingly, both the level and the distribution of the methyl-esters on the HG backbone are key features in determining pectin's functionality [6, 7]. The RG-I region of pectin is composed of the dimer repeat α-1,4-d-galacturonosyl-α-1,2-l-rhamnose which are decorated with arabinose and/or galactose rich neutral sugar side chains attached to O-2 of the rhamnose residue [1, 8].

Pectins are widely extracted from fruit and vegetable by-products, such as citrus peel, apple pomace or sugar beet pulp, yielding pectins with high GalA contents (up to 70-75%), a wide range of methyl-esterification [1, 9], and generally, low levels of RG-I segments (5-10%) [1]. Commercial pectins are widely used as a stabilizer, thickener and even emulsifier by the food industry [9]. Pectin as a food ingredient, and/or as part of fruits and vegetables, represents one of the most widespread dietary fibers in human nutrition.

By consuming foods containing dietary fibers such as pectin, both the immune system as well as the colonic microbiota can be beneficially influenced [10]. Only a few studies investigate the effects of different fibers on gut microbiota in different intestinal segments. Each intestinal segment has different anatomy and function [11] and the colonization of microbiota also changes between the different intestinal segments [12]. The microbiota composition of small intestine holds mainly facultative anaerobes, while the large intestine contains primarily strict anaerobes [12]. Regarding microbial function, the small intestinal microbiota mainly assists immunity, while the

large intestinal microbiota plays a role in fiber fermentation [7, 13, 14]. Furthermore, small intestinal microbiota is driven by the fast uptake and conversion of simple carbohydrates and helping digestion, while that of colon is driven by specificity of complex carbohydrate conversion [14].

In the gastrointestinal tract, dietary fibers such as pectins are indigestible by human digestive enzymes. However, they can be easily fermented by resident microbiota in the large intestine, thereby influencing its composition and contribution to the production of short-chain fatty acids (SCFA) [14-17]. SCFA can be produced upon fermentation of carbohydrates, while other bacterial metabolites such as indole derivatives are produced upon fermentation of amino acids such as tryptophan. These fermentation products can enhance immune function by inducing mucosal proliferation and strengthening the intestinal barrier integrity [15, 18, 19]. The indole derivatives act through the aryl hydrocarbon receptor (AhR) and are considered to be an important regulator of immune responses [20]. AhR affects the differentiation of anti-inflammatory T regulatory cells (Tregs) [21] that can maintain immune homeostasis and prevent the aggravated immune responses against pathogens [22]. Activation of AhR by ligands has immune barrier protective effects [23] and promote a strong gastrointestinal immune barrier which is essential to prevent pathogens from entering the external environment to the bloodstream [24, 25]. To get an impression of the possible presence of indole derivatives, AhR activation can be measured during fermentation of dietary fibres [26].

Most effects of dietary fibers on the immune system are primarily credited to their beneficial effects on the host's intestinal microbiota, however research on the impact of pectins is very limited. In some studies the microbiota-modulating effects of pectin have been confirmed *in vivo* in rodents such as in rats and mice [27-29], but also in piglets [30] and in humans [31]. There are also human studies that show a very minor or no effect of pectin supplementation [32]. Tian et al. [27] have found that supplementation of 3% (w/w) of the total feed with either Low or High Methyl-esterified pectins (LM and HM pectin respectively) modulates the level and location of colonic fermentation of dietary fibers, and consequently the on-site microbiota composition and SCFA level and composition in rats. Till now the impact of pectins on the intestinal microbiota has been studied for several pectic polysaccharides. However, the significance of the structural properties of pectins regarding the effects on microbiota is still unclear.

In addition to pectin's bioactivity through its fermentation metabolites, pectin can also initiate beneficial immune effects through a direct interaction with intestinal epithelial and immune cells [10, 13]. Recent studies suggest that the functional properties of pectins and pectic oligosaccharides

in the intestine depend on their chemical characteristics, such as DM or DB [10, 27, 30]. Moreover, Sahasrabudhe, Beukema et al. [6] demonstrated that low DM pectins in 3 mg/day dose have anti-inflammatory properties through the direct binding to and inhibition of Toll-like receptor 2 (TLR2) in mice. Furthermore, Beukema et al. showed that pectins having a high DM and a high DB at the same time, also inhibit TLR2 and TLR1 dimerization in cell lines which is required for immune response and showing the importance of distribution of methyl-esters within pectins [33]. Moreover, it has been also demonstrated that those pectins given at such a low dose induce an anti-inflammatory effect in mice with mucositis [34].

In this study, we investigated the fermentation and structure-function relationship of pectins in full detail in healthy mice with the aim to understand the impact of pectin in different parts of the intestine, *i.e.* the duodenum, jejunum, ileum, cecum, proximal- and distal colon. To this end the diets of the mice were supplemented for 1 or 4 weeks with four pectins differing in DB and DM at a dose of 3 mg/day (0.1%). In a previous study, these pectins were found to impact the immune system in a structure-dependent way [33] which we aim to explain by the new observations in this current study. We focus on the correlation between the structure of the pectins, the microbiota composition, SCFA production in different segments of the intestine and the previously observed immune effects.

2. Materials and methods

2.1. Pectins

Commercially extracted lemon pectins having a different level and distribution of methyl-esters were provided by CP Kelco (Copenhagen, Denmark). Two low DM pectins, DM18 having a low degree of blockiness (LB) and DM19 having a high degree blockiness (HB), and two intermediate DM pectins, DM43 (HB) and DM49 (LB), were used in this study. Molecular weight, sugar composition, DM and DB have been analyzed and described previously (Table 1 & Figure S1) [33].

2.2. Experimental animals and sample collection

C57BL6 female mice (10 weeks old) were obtained from Janvier Laboratories (Le Genest-Saint-Isle, France). The experimental use of animals was approved by the Animal Ethical Committee of the University of Groningen (CCD application number AVD1050020171487). Mice were cohoused with a total number of five mice in individually ventilated cages. Animals were given *ad libitum* access to sterilized tap water and RMH-B feed (AB Diets, Woerden, The Netherlands) [6, 35]. Dietary fiber sources of RMH-B feed contain whole wheat (42.3%), and smaller amounts of wheat middling (8.3%), whole oat (8.3%) and maize (in minor amounts). In terms of animal feeds, whole oats are frequently used including the hull containing up to 35% arabinoxylans, consequently, arabinoxylan can also be present in the feed [35, 36].

After 1.5 weeks of acclimatization in the animal facility, mice received pectins in addition to the feed for 1 or 4 weeks. In each group there were 10 mice. Pectins were given twice a day through oral gavage in a volume of 250 µl per gavage (6 mg/ml). The supplemented amount of pectin was approximately 0.1% of the total food intake and about 1% of the total fiber intake. Control mice received 250 µl of sterile water. Animals were anesthetized using isoflurane/O_2 and sacrificed by cervical dislocation. Digesta from duodenum, jejunum, ileum, cecum, proximal- and distal colon was collected for the determination of SCFA and branched-chain fatty acids (BCFA) present, for microbiota analysis and quantification of AhR activating components. Due to the very low amount of digesta in mice, the digests from the same intestinal segment of mice in the same treatment group and same time point were pooled (n=10). While pooling each intestinal content was weighted in the same amounts.

2.3. Analysis of organic acids

Organic acids, including SCFA and BCFA were measured in the mice digests from the duodenum, jejunum, ileum, cecum, proximal- and distal colon. Between 20–150 mg pooled digest was dissolved in 200 µL nuclease-free water, mixed, and consequently centrifuged (20,000× g for 10 min at 4 °C). After mixing and centrifugation, 100 µL supernatant was transferred to vials and used for analysis. SCFA were quantified using a Dionex Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA). Ten µL sample was injected to an ion-exclusion Aminex HPX-87H column (7.8×300 mm) combined with a guard column (Bio-Rad, Hercules CA, U.S.A.). The elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). The column temperature was kept at 65 °C. Elution was done with a flow rate of 0.6 mL/min using 5.0 mM H_2SO_4 [37]. Standard solutions of lactic acid, succinic acid, acetic acid, propionic acid, butyric acid, isovaleric acid and isobutyric acid were prepared in concentrations of 0.05–3 mg/mL. Data was processed using Chromeleon 7.2 (Dionex). SCFA concentrations were expressed as µmol/mg dry matter. Dry matter content was determined by drying the samples in an oven overnight at 60 °C.

2.4. Digesta Samples and Microbiota Profiling

Mice digests from duodenum, jejunum, ileum, cecum, proximal- and distal colon were collected from 200 animals, and immediately stored at −80 °C. Microbiota composition was determined by sequencing barcoded 16S ribosomal RNA (rRNA) gene amplicons using Illumina Hiseq2500 (2 × 150 bp). DNA was isolated using Repeated-Bead-Beating [38] and purified using the Maxwell® 16Tissue LEV Total RNA purification Kit Cartridge (XAS1220). The V4 region of 16S rRNA gene was amplified in triplicate using primers and fecal DNA as a template. Each 35 µL reaction contained 0.7 µL 20 ng/µL DNA template, 7 µL 5 × HF buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 0.7 µL of 10 mM dNTPs (Thermo Fisher Scientific), 0.35 µL DNA polymerase (2 U/µL) (Thermo Fisher Scientific), 25.5 µL nuclease-free water (Promega, Madison, WI, USA), and 0.7 µL 10 µM of sample-specific barcode-tagged primers. Cycling conditions were as follows: 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 50 °C for 10 s, 72 °C for 10 s, with a final extension of 7 min. at 72 °C. Subsequently, the triplicate PCR products were pooled for each sample, purified with the CleanPCR kit (CleanNA, Waddinxveen, The Netherlands), and quantified using the QubitTM dsDNA BR Assay kit (Invitrogen, Eugene, OR, USA). An equimolar mix of purified PCR products was prepared and sent for sequencing (GATC-Biotech, Konstanz, Germany).

2.5. Microbiota Data Analysis

The 16S rRNA gene amplicon sequencing data were processed and analyzed using the NG-TAX 2.0 pipeline, with default settings and R version 3.6.1 [39]. Amplicon sequence variants (ASVs) with less than 0.1% relative abundance were removed. The taxonomic assignment was performed with a threshold of 80%.

Alpha-diversity of the microbiota was determined by calculated at ASV level the richness (Faith's phylogenetic diversity; PD) and diversity (inverse Simpson diversity) indices using respectively the *phyloseq* [40] and *picante* [41] R packages. The variation in microbiota composition between the different digesta was calculated using weighted Unifrac distances [42] based on the relative abundances at ASV level using the *ordinate* function in the *phyloseq* R package. Principal Coordinate analysis (PCoA) was applied to visualize the output, using the function *plot_ordination* in the *phyloseq* R Package. The function *plot_ordination* (*type: taxa*) was also used to extract the species scores and determine their contribution to the principle coordinates. The obtained x- and y- coordinates were used to plot taxa vectors on the PCoA plot using the function *geom_segment* in the *ggplot2* R package [43]. Only the most abundant taxa were visualized as determined by the function *core* in the *microbiome* R package [44].

Distance-based redundancy analysis (dbRDA) [45] was performed to assess the contribution of the explanatory variables (organic acids, AhR activation and double-positive Tregs) to the variation in microbiota composition at ASV level between the different digesta. Weighted and unweighted UniFrac distance matrices were calculated using the function *distance* in the *phyloseq* R package. Subsequently, the dbRDA analysis was performed using the function *capscale* in the *vegan* R package [46]. After scaling, the relevant explanatory variables were selected by applying the function *ordistep* in the *vegan* R package with both forward and backward stepwise inclusion. The output of the dbRDA analysis was visualized using the function *ggplot* in the *ggplot2* R package with the significance of the canonical axes tested by the function *anova* in the *vegan* R package. Adjusted R^2 was calculated using the function *RsquareAdj* in the *vegan* R package to determine the unbiased estimators of explained variation, taking into account the number of explanatory variables in the model. Arrows presenting the explanatory variables were plotted by the function *geom_segment* in the *ggplot2* R package. The best-fitting ASVs were selected and plotted by the function *ordiselect* in the *goeveg* R package [47] and the function *geom_segment* in the *ggplot2* R package.

2.6. AhR activation assays

The AhR activation assay was performed as described by Beukema et al. [26]. To study whether caecal samples contained AhR activating components, AhR activation assay was performed using HT29-Lucia™ AhR Cells (Invivogen, Toulouse, France) expressing endogenous AhR. These reporter cell lines express the secreted Lucia luciferase reporter gene. Briefly, AhR-expressing cells were cultured in DMEM culture media (Lonza, Basel, Switzerland) containing 10% dFCS, 50U/ml Penicillin (Sigma, St. Louis, MO, USA), 50 µg/ml Streptomycin (Sigma), 100 µg/ml Normocin (Invivogen). The reporter cells were cultured for three passages before they were maintained in a selection medium containing 100 ug/ml Zeocin (Invivogen).

Cells were seeded at 50.000 cells/well in a 96 well plate (Costar) and incubated for 24 hours. Next, cells were stimulated with caecal digesta samples (20 µg/ml). FITZ (50 µg/ml) was used as positive control and unstimulated cells were used as a negative control. After 24 hours of incubation, 20 µl of supernatant was added to 50 µl of LuciaGold substrate, and bioluminescence (488 nm) was directly measured after addition using a bioluminometer (Varioskan, Thermo Fisher Scientific). Experiments were performed at 37°C and 5% CO_2.

2.7. Statistics

Statistical analysis on results was performed using Graphpad Prism program (La Jolla, CA, USA). Normal distribution of AhR data was confirmed using the Kolmogorov-Smirnov test. Data that were not normally distributed were log-transformed before analysis. Values that are normally distributed, are expressed as mean ± standard deviation (SD). Statistical comparisons were performed using two-way ANOVA. Post-testing between control and different pectins was performed with Tukey post-test. The significance of the dbRDA canonical axes tested by the function *anova* in the *vegan* R package.

3. Results

3.1. Structural characteristics of pectins fed to the mice

The studied pectins were previously characterized for GalA content, monosaccharide composition, molecular weight distribution, degree of methyl-esterification and degree of blockiness as summarized in Table 1 [33]. All pectins were mainly composed of GalA (≥95 mol%). The pairs of pectins were chosen because they have different degrees of methyl esterification (DM). Each pair has a similar DM and similar features regarding sugar composition and molecular weight, but differ in the distribution of methyl-esters over the pectin's backbone as illustrated by their DB and absolute degree of blockiness (DB_{abs}), especially for the intermediate DM pectins. Pectins having a low degree of blockiness and consequently having a more random distribution of methyl-esters are depicted as LB pectin and pectins having a high degree of blockiness are referred to as HB pectins.

Table 1. *Constituent monosaccharide composition (mol%), carbohydrate content (w/w%), molecular weight (Mw) and DB and DB_{abs} of pectins. Table modified after [33].*

Pectin/DM[a] (%)	Origin	DB[b] (%)	DB_{abs}[c] (%)	MW[d] (kDa)	Monosaccharides (mol%)[e]					Total Carbohydrate (w/w%)
					Rha	Ara	Gal	Glc	GalA	
DM18	lemon	86	70	78	1	0	2	0	97	62
DM19	lemon	94	76	75	1	1	3	0	95	63
DM43	lemon	60	36	79	0	0	0	0	99	77
DM49	lemon	33	16	114	0	1	2	0	96	73

[a] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample.
[b] Degree of blockiness (DB): the amount of mono-,di- and triGalA per 100 mol of the non-esterified GalA in the sample.
[c] Absolute degree of blockiness (DB_{abs}): the amount of mono-,di- and triGalA per 100 mol of the total GalA in the sample.
[d] Molecular weight (Mw) as measured by HPSEC
[e] Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, GalA = Galacturonic Acid.

3.2. Altered alpha-diversity of microbiota upon pectin supplementation

The impact of the supplementation of the different pectins to the diet of mice on the gut microbiota was studied focusing on the microbiota variations between different gut segments of healthy mice. High alpha diversity is often associated with enhanced resilience to diseases and better homeostasis [48].

Overall, the alpha-diversity indices and microbiota variability of the large intestine are higher than in the small intestine (Figure 1). No major differences were observed in the InvSimpson diversity index in the different segments of the small intestine neither after one week nor four weeks, neither between different diets after pectin supplementation (Figure 1A, C). In contrast, in the different segments of the large intestine the InvSimpson diversity index was somewhat higher for the pectin fed groups compared to the control group at both time points. The highest diversity was shown for the DM19 (HB) pectin supplemented group both after 1 and 4 weeks specifically in the colon. While the lowest diversity was shown for the control diet.

The small intestinal bacterial community had a lower richness, than the large intestinal bacterial community (Figure 1B, D). The Faith's phylogenetic diversity index of the small intestinal microbiota is shown to be the highest in the duodenum, and lowest in the ileum, but there is no clear diet-induced effect. The highest Faith's phylogenetic diversity index in the colon was found for the DM19 (HB), DM 43 (HB) and DM49 (LB) groups. Based on the Faith's phylogenetic diversity index, the lowest diversity in the large intestine was observed after the first and fourth weeks for the control and DM18 (LB) fed mice. This indicates that specific phylogenetic lineages may be influenced by the different pectin structures.

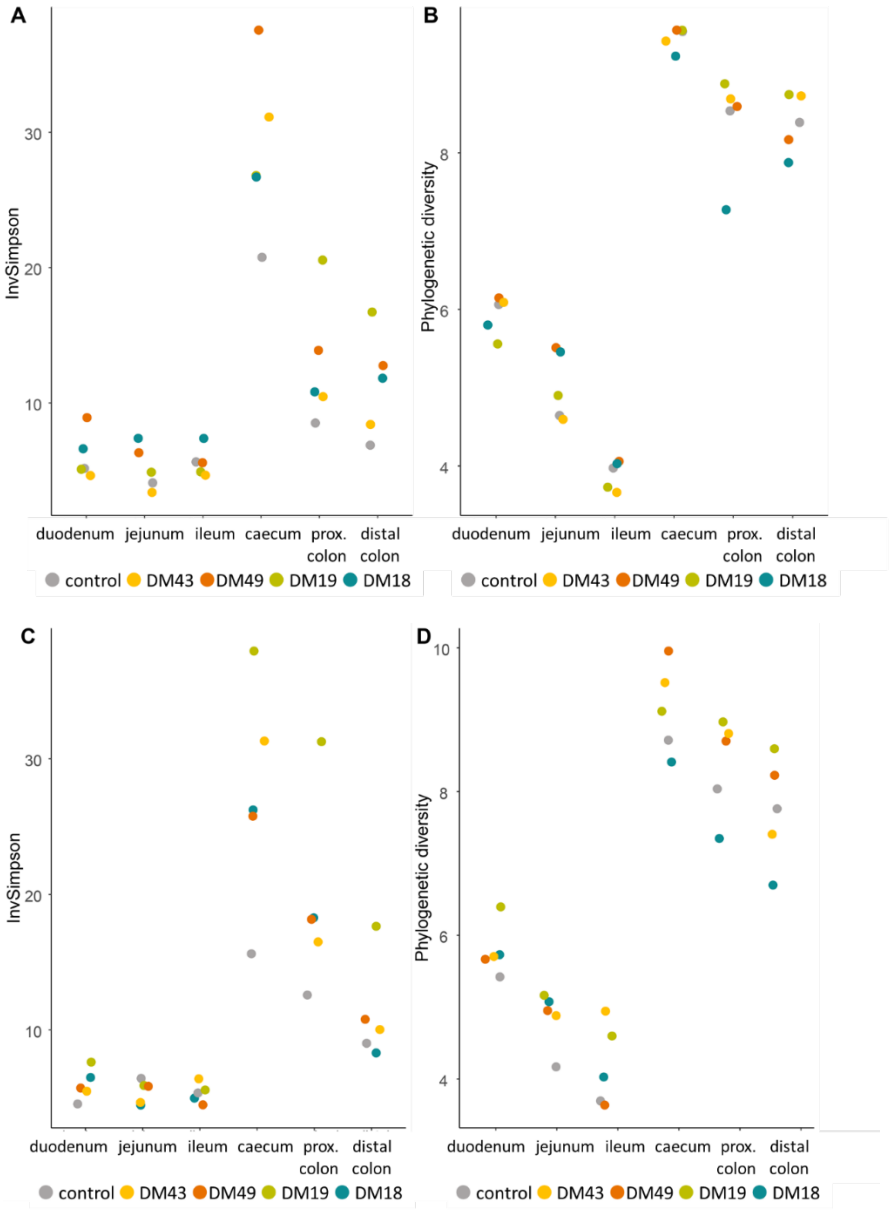

Figure 1. *Alpha-diversity of microbiota composition of digesta collected from the duodenum, jejunum, ileum, caecum, proximal- and distal colon of mice fed with structurally different pectins using inverse Simpson (A, C) and Faith's phylogenetic diversity (B, D) after 1 week (A, B) and 4 weeks (C, D) of supplementation.*

3.3. Differences in microbiota composition between intestinal segments

The microbiota composition across the different intestinal segments was determined to monitor how the different diets affect PCoA based on weighted UniFrac distances revealed distinct clustering patterns between the different gut segments. Figure 2 demonstrates that the variations between the small intestine and large intestine explain 84% of the microbial differences in the most abundant microbes, and that 7.2% of the variation is explained by the differences between the caecum and the colon segments.

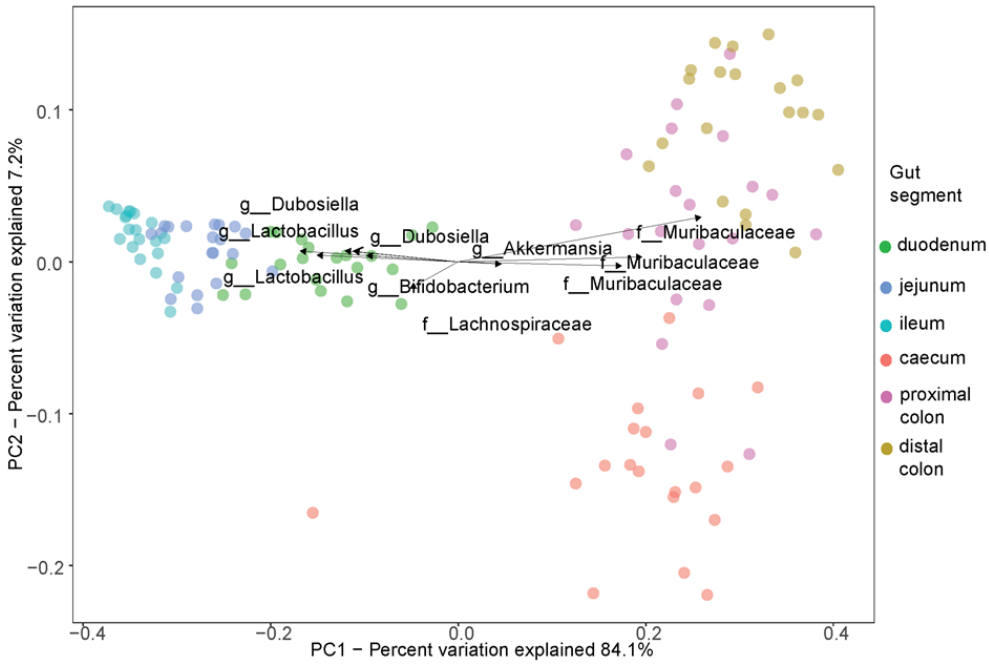

***Figure 2**. PCoA analysis using weighted UniFrac distances of microbiota composition in digesta collected from duodenum, jejunum, ileum, caecum, proximal- and distal colon, including all diets and time points. The contribution of the most abundant bacteria to PC1 and PC2 is presented by the direction and length of the taxa arrows.*

As expected, duodenal, ileal and jejunal microbiota compositions are quite different from caecal and colonic microbiota composition (Figure 3). Firmicutes (*Lactobacillus, Lachnospiraceae* and *Dubosiella, Lachnoclostridium*) were most abundant in the small intestine. In the large intestine, next to the likewise highly abundant Firmicutes, also Bacteroidetes (*Muribaculaceae, Alloprevotella and Prevotellaceae*) were highly abundant. Next to them in in all intestinal segments Actinobacteria (*Bifidobacterium* and *Enterorhabdus*), Proteobacteria and Verrucomicrobia (*Akkermansia*) were present (Figure 3, S4 and S5).

Moreover, a closer inspection of figure 3 also revealed diet-dependent differences. Actinobacteria was highly present in the control and DM18 (LB) supplemented groups particularly after 4 weeks, while in other diet groups Actinobacteria was only present in low abundance. Proteobacteria were most abundant in the DM19 (HB) fed mice, but were also present in the control, DM43 (HB) and DM49 (LB) groups at both time points. Verrucomicrobia was more abundant in the pectin fed animals, and by the fourth week there was an increase of Verrucomicrobia, but only for the DM19 (HB) pectin group.

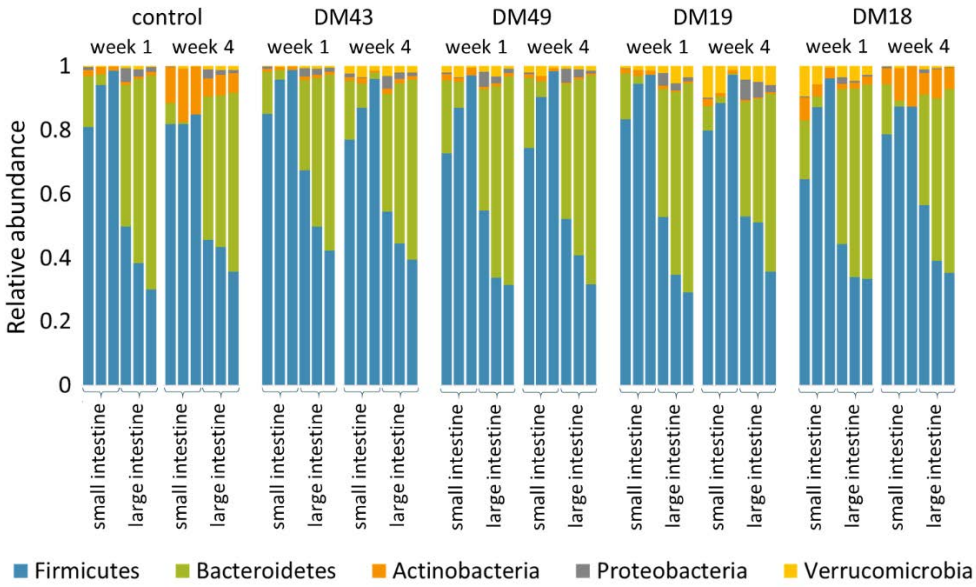

Figure 3. *Relative abundance of most predominant phyla based on the average relative abundance across the entire dataset in the small intestine (in the order of duodenum, jejunum, ileum) and large intestine (in the order of caecum, proximal-, and distal colon) of control mice and pectin fed (DM43, DM49, DM19 and DM18) mice after 1 and 4 weeks. Phylum are listed in the legend. Each column represents a given type of sample from 10 pooled mice intestine segment digesta per time point.*

3.3.1. Diet-dependent effects on the microbiota composition

Small intestine

In the small intestine, only minor diet-induced differences in microbiota composition were observed after 1 and 4 weeks of control diet or pectin supplementation. However, zooming in on the relative abundance of the bacteria at genus level, some differences can be observed for the small intestinal microbiota (Figure 4). In general *Lactobacillus* was the most

dominant in all small intestinal segments, followed by *Dubosiella* and Muribaculaceae.

At the first week the duodenum of the control group was dominated by *Lactobacillus, Dubosiella* and Muribaculaceae and the same trend was observed for the other groups except for the DM18 (LB) fed mice, where a high abundance of *Bifidobacterium* and *Akkermansia* was found. In the jejunum and ileum of control mice there was a steady incline in the dominance of *Lactobacillus* and *Dubosiella* for all groups, while the relative abundance of Muribaculaceae slowly declined, and by reaching the ileum the presence was overruled by other bacteria.

After four weeks of supplementation, similar trends were seen as the first week of control group, but only for the DM43 (HB), DM49 (LB) and DM19 (HB) groups. Besides the *Lactobacillus, Dubosiella* and Muribaculaceae, Lachnospiraceae became more abundant in all groups, and the two low DM, DM18 (LB) and DM19 (HB) pectin supplemented groups had the highest abundance of all groups. The control and the DM18 (LB) groups had the dominance of *Lactobacillus* and *Dubosiella* along the small intestine, while *Bifidobacterium* appeared in high abundance compared to the other groups, where it was not present. Furthermore, DM19 (HB) group had a higher dominance of *Akkermansia* in the duodenum and jejunum, but not in the ileum.

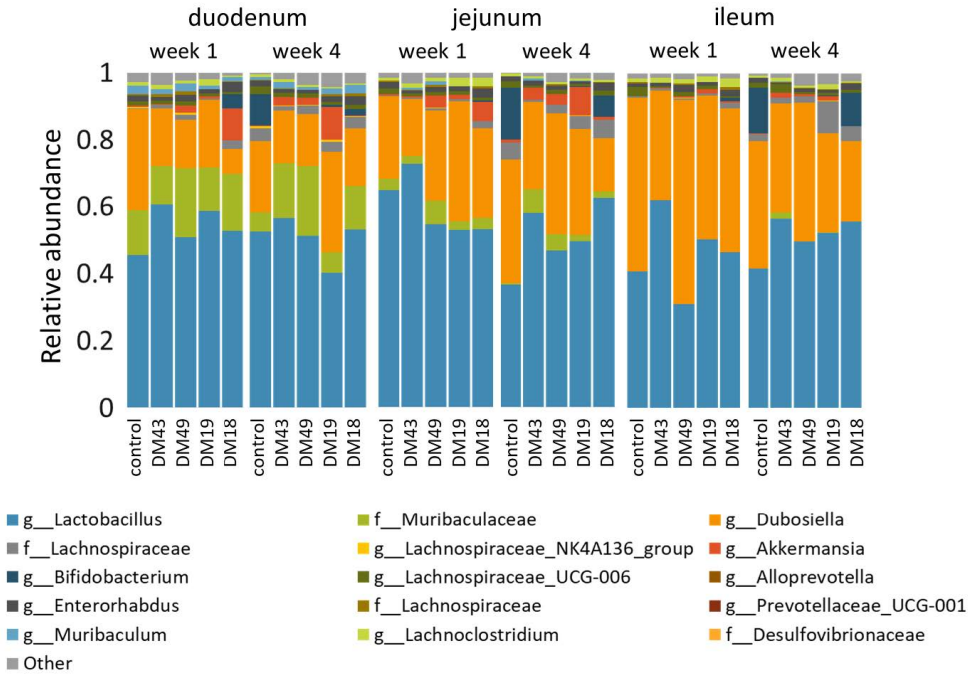

Figure 4. *Relative abundance of most predominant bacteria (top 15, ranked based on the average relative abundance across the entire dataset) in the small intestine (duodenum, jejunum, and ileum) of control mice and pectin supplemented (DM43, DM49, DM19 and DM18) mice after 1 and 4 weeks. Top 15 microbial families and genus are listed in the legend. Other bacteria are summarized as "Other". Each column represent the pooled intestine segment per time point of 10 mice.*

Large intestine

In the large intestine the microbial differences were highly driven by the intestinal segments both after 1 and 4 weeks of control diet or pectin supplementation. The microbiota from the caecum, proximal- and distal colon was also analyzed at genus level as shown in figure 5. The large intestinal microbiota of both control and pectin fed mice was in general dominated by Muribaculaceae, *Lactobacillus*, *Dubosiella* and Lachnospiraceae.

After one week, caecal microbiota of DM43 (HB), DM49 (LB) and DM19 (HB) groups next to the abundant bacteria (Muribaculaceae, *Lactobacillus*, *Dubosiella* and Lachnospiraceae), *Lachnospiraceae NK4A136* was shown to be present in 2-3 times higher relative abundance than in the caecum of control group. In the caecum of all pectin fed groups *Alloprevotella* and *Prevotellaceae UCG-001* was also found to be 2-4 times more abundant than in the caecum of control mice. Continuing along the large intestine in the

proximal- and distal colon in all groups the relative abundance of Firmicutes, such as *Lactobacillus* and *Dubosiella* decreased, and at the same time, the relative abundance of Bacteroidetes such as Muribaculaceae were increased when reaching the end of the large intestine. Interestingly, after 1 week the relative abundance of *Akkermansia* was the highest for the DM18 (LB) and DM19 (HB) pectin supplemented diet groups. In the caecum of control mice at week 4, the relative abundance of *Lactobacillus* increased, while in the pectin fed animals the levels of *Lactobacillus* were similar to the first week. In the caecum of DM43 (HB), DM49 (LB) and DM19 (HB) fed mice, *Lachnospiraceae NK4A136* were present in a similar high abundance than after 1 week. However, *Alloprevotella* and *Prevotellaceae UCG-001* were only highly abundant for DM43 (HB), DM49 (LB) and DM19 (HB) groups and not for the DM18 (LB) group anymore. What stood out is the high dominance of *Bifidobacterium* in the caecal digesta of only control and DM18 (LB) fed mice similarly as already observed in the small intestinal segments of these diets. The caecal microbiota of the DM49 (LB), DM43 (HB) and DM19 (HB) groups presented a higher relative abundance of *Akkermansia* after 4 weeks, in contrast to the control and DM18 (LB) pectin groups.

After 4 weeks, in the proximal and distal colon the relative abundance of *Lachnospiraceae NK4A136* had declined compared to the amounts in caecum. However, it was still 2-3 fold higher in the DM43 (HB), DM49 (LB) and DM19 (HB) groups, while in the control and DM18 (LB) groups it was almost diminished. The abundance of *Akkermansia* has increased by the fourth week only in the DM43 (HB), DM49 (LB) and DM19 (HB), while for the colon of DM18 (LB) mice, it has diminished. In the colon of DM43 (HB), DM49 (LB) and in particularly DM19 (HB) groups, the relative abundance of *Desulfovibrionaceae* in week 4 was shown to be much higher, compared to control and DM18 (LB) groups. On the other hand, the DM18 (LB) pectin supplemented diet group continued to have a high abundance of *Bifidobacterium* present after 4 weeks of supplementation as it was shown in all segments and time points for the DM18 (LB) group.

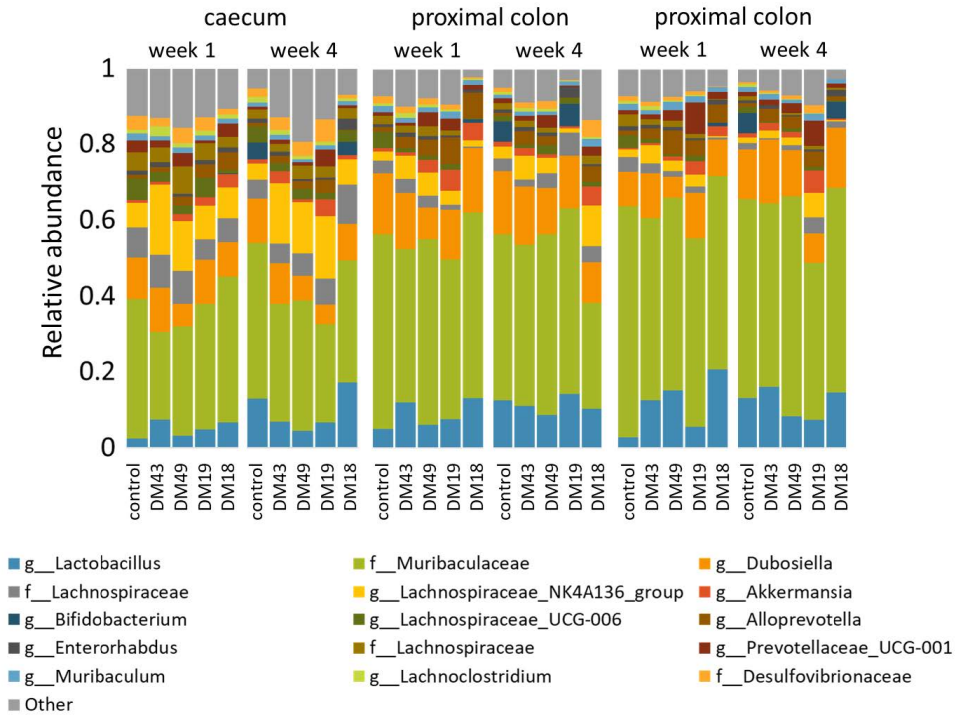

Figure 5. *Relative abundance of most predominant bacteria (top 15, ranked based on the average relative abundance across the entire dataset) in the large intestine (caecum, proximal colon and distal colon) of control mice and pectin supplemented (DM43, DM49, DM19 and DM18) mice after 1 and 4 weeks. Top 15 microbial families and genus are listed in the legend. Other bacteria are summarized as "Other". Each column represent the pooled intestine segment per time point of 10 mice.*

3.4. Fermentation metabolites and AhR activation in mice digesta

Total organic acid concentrations in the small intestine (duodenum, jejunum, ileum) and large intestine (caecum, proximal- distal colon) were analyzed both for the control animals and mice fed with pectin supplemented diets after week 1 and week 4 (Figure 6). Overall, the amount of acids was the highest in the duodenum and caecum. Interestingly the total amount of organic acids in the small- and large intestine between 1 and 4 weeks of pectin supplementation decreased, but not in all cases. The supplementation of pectins did not have a major impact on the SCFA profiles (Figure 6 and S2).

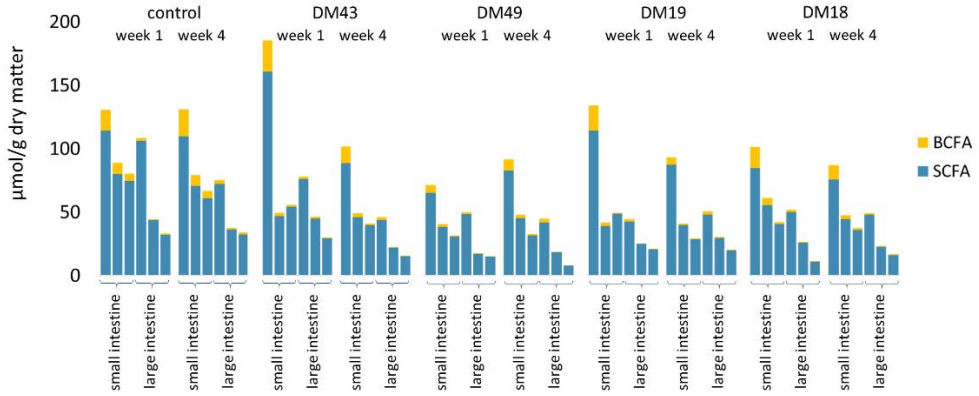

Figure 6. *Amount of SCFA and BCFA found in the small intestine (in the order of duodenum, jejunum, ileum) and large intestine (in the order of caecum, proximal-, and distal colon), collected from control mice or from mice fed a diet supplemented with DM18, DM19, DM43 or DM49 pectins. SCFA and BCFA are expressed in µmol/g dry matter from the collected and pooled gut segments per diet group, after week 1 and week 4 of receiving the diet.*

In the small intestine, the control group showed minor changes in SCFA and BCFA after 4 weeks compared to the first week. For the pectin groups, the intermediate DM pectin (DM43 (HB) and DM49 (LB)) supplemented diet groups showed somewhat higher cumulative SCFA production in the small intestine compared to the control group, but only after the first week of supplementation. The low DM pectin (DM18 (LB) and DM19 (HB)) fed mice produced fewer SCFA and BCFA compared to the control and intermediate DM pectin fed animals both after 1 and 4 weeks (Figure 6).

In the large intestine, the total amount of SCFA and BCFA were decreased for all pectin fed mice compared to the control group already after the first week, as the pectins might have affected the absorption of SCFA and BCFA (Figure 6). The DM43 (HB) pectin fed mice showed the highest SCFA concentration of all pectin supplemented groups (105 µmol/g) compared to the other pectin supplemented groups (<78 µmol/g), but still lower than in the control group (143 µmol/g). Acetic acid was the most abundant SCFA formed in the caecum and both proximal and distal colon for all mice, followed by butyric acid and propionic acid (Figure S2). In contrast to the SCFA, there was hardly any BCFA present in the large intestine (Figure 6 and S2).

In addition to the SCFA and BCFA, other fermentation metabolites could have been formed, such as indole derivatives which can activate AhRs. AhR activation was measured for the caecal samples of mice fed with the

structurally different pectins. This was done for caecal samples only as most AhR receptors are found in this part of the intestine and as the cecum is the primary site for generation of immunity, and it reflects the entire intestinal tract [26]. Figure 7 illustrates that AhR was mainly activated by the digesta of mice whose diet was supplemented with DM19 (HB), DM43 (HB) and DM49 (LB) pectins, especially after 4 weeks of supplementation. This suggests that fermentation of specific pectin structures leads to the production of indole derivatives which were able to activate AhR.

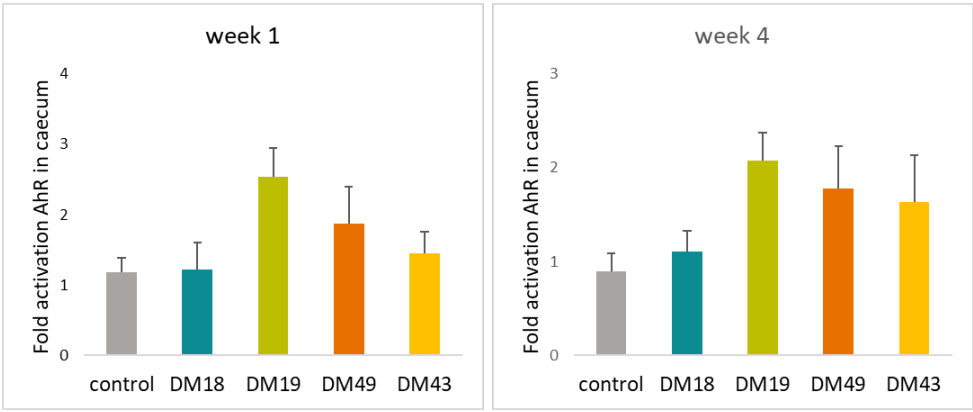

Figure 7. *Activation of the AhR, which gives an indication of the presence of AhR ligands in the digesta. The possible presence of indole derivatives in caecal digesta samples was measured by the activation of an AhR reporter cell line. Caecal digesta from control mice or from mice supplemented with DM18 (LB), DM19 (HB), DM49 (LB) or DM43 (HB) pectins were tested.*

3.5. Variation in microbiota composition linked to AhR activation, Tregs and organic acids

Pectins can interact indirectly with the immune system by the modulation of the intestinal microbiota and microbial-derived metabolites such as AhR ligands. It has been analyzed whether the AhR activation can be found in caecum as most of the receptors are in the caecum and it is the main site for the generation of immunity, representing the entire intestinal tract (Figure 7), double-positive T regulatory cells (Tregs), measured in mesenteric lymph nodes by Beukema et al. [26] (Figure S3) and organic acids (Figure 6 and S2) were linked to diet-induced differences in microbiota composition using distance-based redundancy analysis (db-RDA).

In the large intestine, the unweighted UniFrac db-RDA showed that after 1 week Tregs are most correlated with the large intestinal segments of DM43 (HB) and DM49 (LB) pectin fed groups and somewhat of the presence of *Lachnospiraceae_NK4A136,* while AhR activation can be associated with *Lachnospiraceae_UCG-006* and the DM19 (HB) pectin supplementation (Figure 8A). Isobutyrate was shown to be somewhat correlated with caecal segments and the presence of Desulfovibrionaceae.

After four weeks each diet formed a cluster, independently from large intestinal segments (Figure 8B). Tregs after four weeks clearly were associated with the presence of *Lachnospiraceae_NK4A136* and Muribaculaceae and with DM43 (HB) and DM49 (LB) pectin groups. AhR activation is primarily associated with DM49 (LB) pectin group.

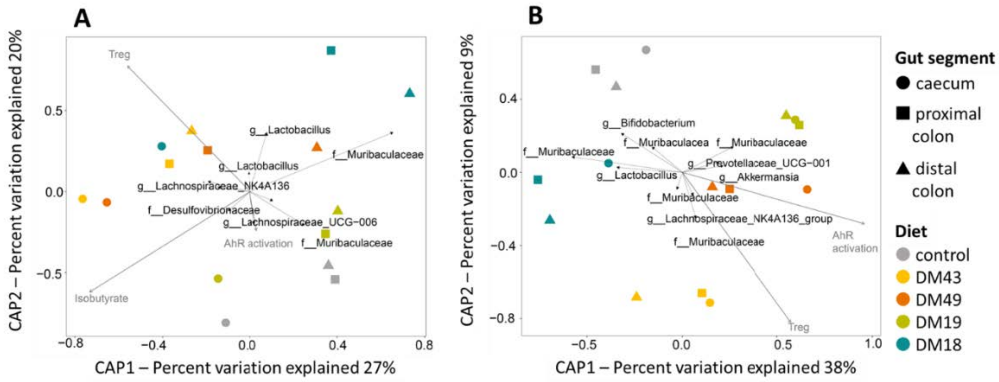

Figure 8. *Redundancy-based analysis of unweighted UniFrac distances of microbiota composition in digesta collected from the caecum, proximal- and distal colon after 1 week* ***(A)*** *and 4 weeks* ***(B)****, of control, DM18, DM19, DM43 and DM49 pectin supplementation. The explanatory variables explained 44% of the variation in microbiota composition, as indicated by the adjusted* R^2 *after one week, and explained 38% after the fourth week. Explanatory variables Treg, AhR and BCFA are shown as grey arrows. Direction and length of the taxa arrows (black) presents the contribution of taxa with best fit to* ***(A)*** *CAP1 (P<0.001) and CAP2 (P<0.01) and* ***(B)*** *CAP1 (P<0.001) and CAP2 (P: not significant)*

After the first week in the large intestinal digesta, the db-RDA weighted UniFrac (Figure 9A) revealed that AhR activation is somewhat correlated with DM19 (HB) and DM 49 (LB) pectins. Isovalerate levels were associated with the DM43 (HB) pectin in the large intestine. While isobutyrate was somewhat correlated with caecal segments and Desulfovibrionaceae, similarly as seen at the unweighted UniFrac. After 4 weeks, control and DM18 (LB) groups were shown to be different from the other groups and associated with *Lachnospiraceae_NK4A136,* Muribaculaceae, *Lactobacillus* and

Bifidobacterium. AhR activation was most associated with *Alloprevotella* and somewhat also with DM19 (HB), DM43 (HB) and DM49 (LB) pectin supplemented groups (Figure 9B).

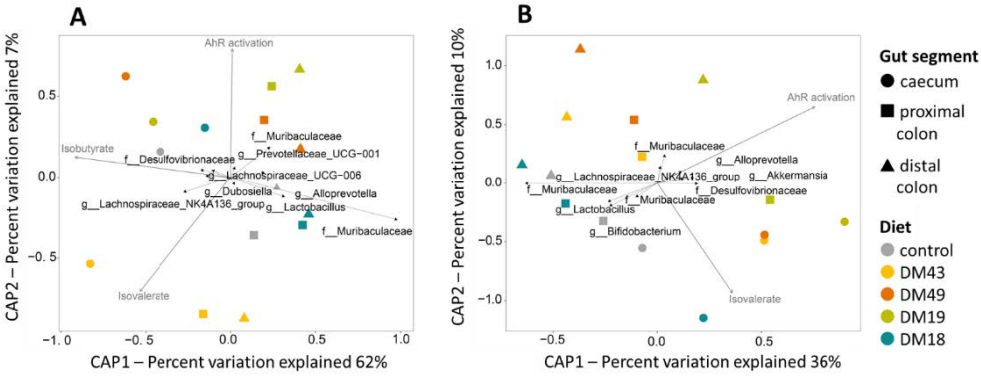

Figure 9. *Redundancy-based analysis of weighted UniFrac distances of microbiota composition in digesta collected from the caecum, proximal- and distal colon after 1 week* ***(A)*** *and 4 weeks* ***(B),*** *of control, DM18, DM19, DM43 and DM49 pectin supplementation. The explanatory variables explained 69% of the variation in microbiota composition, as indicated by the adjusted R^2 after one week, and explained 39% after the fourth week. Explanatory variables Treg, AhR and BCFA are shown as grey arrows. Direction and length of the taxa arrows (black) presents the contribution of taxa with best fit to* ***(A)*** *CAP1 (P<0.001) and CAP2 (P not significant) and* ***(B)*** *CAP1 (P<0.01) and CAP2 (P: not significant)*

An interesting aspect was that next to the AhR activating indole derivatives, BCFA isovalerate and isobutyrate were also shown as explanatory variables, mainly associated with the caecum samples. As the differences were more distinct in the unweighted UniFrac, this observation indicates that the differences in microbiota profile are mainly driven by the presence/absence of bacterial taxa rather than relative abundance, meaning that less abundant bacteria together were more responsible for the seen differences than the few highly abundant bacteria.

4. Discussion

In the present study, the effects of the supplementation of a very low dose (1% of total fibers in the diet) of structurally different pectins were compared in different segments of the intestine in healthy mice. To the best of our knowledge it has been shown here for the first time how low doses of pectins in diet affect microbiota composition and SCFA in different parts of the intestine.

The environmental and functional difference of the various intestinal segments affect the colonization of gut microbiota [12]. The composition of microbiota is normally rather stable and consists of both permanent core taxa and transient bacterial taxa which play a role in gut health and disease [49].

Interestingly, the pectins included in this study impacted the microbiota composition in the different gut segments in a structure-dependent way. The small intestine accommodates a highly complex microbiota, although with much less diversity and abundance (10^3 –10^7 bacterial cells per gram) compared to the colonic microbiota (10^{12} bacterial cells per gram) [50, 51]. Although having a low diversity and abundance, the small intestinal microbiota might affect the large intestinal microbiota [50-53]. In our study it has been found that the small intestinal microbiota, in general, was dominated by *Lactobacillus, Muribaculum* and *Dubosiella* which is in accordance with previously reported literature [52, 54]. In addition, in the groups of control mice and DM18 (LB) pectin fed mice the relative abundance of *Bifidobacterium* was high after 4 weeks, while in other groups it was only present in the intestine of DM18 (LB) pectin fed mice already after one week, but not in other groups.

The large intestine, compared to the small intestine is less influenced by transient bacteria, furthermore it provides a better environment for bacterial growth [54]. In the large intestine (caecum, proximal- and distal colon) the microbiota composition was clearly different from the small intestinal microbiota, with a higher alpha diversity and with Muribaculacea and Lachnospiraceae being most dominant in the large intestine which is also in accordance with literature [54]. A higher relative abundance of bacteria being part of the Ruminococcaceae family previously reported as dietary fiber- or pectin-degraders [27, 55, 56] was observed The highest alpha diversity was observed in the caecum (Figure 1). Alpha diversity shown by the diversity indices is increased upon pectin supplementation, showing that the complex pectin molecules stimulate a broad range of cooperating bacteria necessary to complete the degradation [16]. Moreover, Lachnospiraceae and Ruminococcaceae are distinctively able to degrade a wide variety of dietary

fibers [55]. In the groups where the diets were supplemented with DM19 (HB), DM43 (HB) and DM49 (LB) pectins, the relative abundances of *Lachnospiraceae NK4A136*, *Alloprevotella*, *Prevotellaceae UCG-001* and *Akkermansia* were higher than in the control and DM18 (LB) groups. Interestingly, we observed already in the small intestinal segments after 4 weeks, in the control and DM18 (LM) pectin fed mice large intestinal digesta *Bifidobacterium* were present in contrast to all the other pectin supplemented groups. An implication of this is the possibility that other microbial groups are more stimulated at the cost of *Bifidobacterium*. On the other hand, previous research has shown that prebiotic-rich diets can increase the number of *Bifidobacterium* and/or *Lactobacillus,* and both pectin and arabinoxylan present in the control diet are potential prebiotics as shown for humans [57, 58]. These literature findings accord with another observation showing that arabinoxylan as present in reasonable amounts in the currently used basal mice diet by the presence of oat hull, can induce a specific increase in *Bifidobacterium* [59]. Furthermore, in previous findings a relationship between DM and fermentability has been reported, where LM pectins were fermented faster both *in vivo* and *in vitro* than HM pectins [16, 27, 60]. It could be argued whether the microbiota differences were due to the pectin supplementation, as it accounts for only about 1% of the total fiber present in the feed. However, the amount of pectin added in a solution through the oral gavage might have changed both the consistency and the fermentability of the feed in the intestine and may have led to local high concentrations of pectin in the intestine.

In the study of Tian et al. [27] rats were fed with low amounts of structurally different pectins mixed in the feed (3% of the total feed, 20% of total fiber), which is seemingly low, but still 20 times higher than the added pectin in our study. Tian et al. demonstrated that rapid fermentation of pectins induced a shift in fermentation of other fibers present in the feed like arabinoxylans, also present in the feed in our study. In their experiment, pectins stimulated *Lactobacillus* and Lachnospiraceae similarly to the DM18 (LB) and DM19 (HB) pectins in our study.

It is increasingly studied how microbial metabolites influence host's physiology [61, 62]. In this study, the most surprising aspect of the metabolite levels in the digesta involves the lower SCFA and organic acid content in the pectin-supplemented mice compared to the acid levels present in the intestinal tract of control animals both in the small- and large intestine. In all the pectin fed, DM18 (LB), DM19 (HB), DM43 (HB) and DM49 (LB) groups 26-46% lower organic acid amounts were found compared to the control diet. This outcome is in line to that of previous studies, where the SCFA levels were not raised upon pectin supplementation in such low concentration (1%)

[6]. Tian et al. [27] have shown that the low DM citrus pectin increased SCFA production, but high DM citrus pectin did not. It seems that the minor amount of pectins and its consequent change of microbiota composition affected the SCFA production.

Even though SCFA levels were not enhanced it is possible that other microbial-derived fermentation metabolites such as indole derivatives were produced instead, since a high activation of AhR was measured after stimulation with caecal digesta from DM19 (HB), DM43 (HB) and DM49 (LB) pectin fed mice [26]. To relate metabolites of microbial fermentation, such as the SCFA, AhR activation and Treg and the variations in microbiota composition to the different diets, the relative abundance of the 15 most abundant bacterial groups were subjected to distance-based redundancy analysis. In the current study it has been found that AhR activating metabolites and also BCFA can be correlated to the different microbiota compositions of DM19 (HB), DM43 (HB) and DM49 (LB) supplemented diet-fed mice, particularly in the large intestine. AhR activating metabolites can be indole derivatives which represent small signaling molecules from plants, or endogenously produced by tryptophan metabolizing bacteria [19, 62]. Indoles by the interaction with the AhR can maintain the gut epithelial barrier and enhance immune activity, while dampening the release of pro-inflammatory cytokines [63]. Among microbial metabolites, indole derivatives are produced by a number of bacteria including Bacteroidetes which were found in the large intestine of mice in this study. Bacteroidetes can enhance the microbial tryptophan metabolism and through that regulate the levels of indole derivatives and activate AhR [64]. Moreover, members of the Lachnospiraceae family are also associated with the production of indole derivatives [62] and Lachnospiraceae are present in higher relative abundance in the large intestinal digesta of DM19 (HB), DM43 (HB) and DM49 (LB) pectin supplemented mice. It is possible that AhR activating microbial metabolites are also responsible for the generation of Tregs in DM19 (HB), DM43 (HB), and DM49 (LB) supplemented mice. This might suggest that not the SCFA, but other microbial fermentation metabolites derived of DM19 (HB), DM43 (HB) and DM49 (LB) pectin fermentation have AhR activating properties. Pectin fermentation might lead to changes in amino acid fermentation, resulting in different levels of the AhR ligands.

A clear effect of DB_{abs} of lower DM pectins on alpha diversity and microbiota composition was found. DM18 (LB) pectin supplemented group was different than the DM19 (HB), but has shown similar low alpha diversity and microbiota composition and DM18 (LB) pectin also induced *Bifidobacterium* just as the control group. The only difference between DM18 (LB) and DM19 (HB) pectins in their structural feature is the pattern of methyl-esterification.

DM18 (LB) has somewhat lower DB than DM19 (HB), 84% and 94% respectively and it is known that the level and pattern of methyl-esterification have an influence on molecular conformation, solubility, viscosity and gelling properties. Higher viscosity can possibly decrease gastric emptying, while it may increase intestinal transit time [65]. Consequently altering digestive and fermentative efficiency, and modulation of substrate availability for the intestinal microbiota for fermentation [61, 66]. The increased viscosity and by that changes in transit time may explain differences in SCFA contents, as well as microbial compositional differences. In particular, transit time is shown to increase the richness of the microbiota and alter the composition of microbial communities [66]. This might have also affected the SCFA production or have caused a more rapid utilization of the organic acids by the richer microbial communities found in the pectin fed groups. The gelling behavior of pectin could possibly also explain the differences in alpha diversity and microbiota composition between DM19 (HB) and DM18 (LB), with the latter being more similar to the control group. Higher DM pectins can form a gel at low pH < 3.5 [67] which pH is provided in the stomach, therefore it is possible that DM43 (HB) and DM49 (LB) pectins have delayed gastric emptying and promoted intestinal transit time which is already possible at 6 mg/ml (0.6 w/v%) pectin concentrations. Previous studies have explored the hydrocolloid and *in situ* gelling properties of pectins and found that low DM pectins can form a strong gel at 1.5 w/v% in rat stomach [68, 69]. Although a 0.6 w/v% pectin solution may not give a (strong) gel yet, it may have an increased viscosity. The mouse stomach volume is about 400 µl and a 250 µl added pectin solution (1.5 mg/250 µl) can already fill the stomach and start gelling there given also the required low pH condition. In the case of the high DB pectins, gels can be formed in the presence of cations such as Ca^{2+} [70], moreover specific pectins may cause an increase in the gelation ability [71]. Among the pectins used, DM19 (HB) is indeed having a high DB and DB_{abs} of 94 and 76 respectively, which can increase the viscosity. These hypotheses suggest that the structurally different pectins might have an effect through their viscosity, which effect may also depend on the method of supplementation. It has been reported that longer transit time is associated with an earlier carbohydrate depletion and by that an increased protein fermentation [66, 72]. In this case, a delayed transit time caused by the viscosity may have increased protein fermentation activity to create the AhR activating metabolites, such as indole derivatives which seem to be correlated to differences in microbiota composition between the different diets.

The different methyl-ester distributions over the pectin backbone may explain the different microbiota stimulating effects for the different pectins. Prior studies have noted the importance of structural differences of pectins

and their effect on the microbial communities, where low and high DM pectins stimulated the microbiota differently [16, 27, 30]. Even similar DM pectins can have completely different patterns of methyl-ester distributions [5] which might explain why the similarly low DM pectins are having different effects.

Our findings, although preliminary, showed that 4 weeks of supplementation with DM19 (HB), DM43 (HB) and DM49 (LB) pectins, changed microbiota composition and especially resulted in an increased microbiota diversity, while stimulating Treg cells and AhRs in a pectin structure-dependent manner.

5. Conclusion

The present study aimed to examine in different parts of the mouse intestine the structure-function relationship of pectins on SCFA production and microbiota composition and to correlate this with previously observed differences in immune effects of pectins.

In our study, the influence of pectins on intestinal microbiota primarily occurred in the caecum and colon. It was demonstrated that in the large intestine DM19 (HB), DM43 (HB) and DM49 (LB) pectins can effectively increase microbiota diversity and also activate AhRs compared to the control diet. In contrast, the control and DM18 (LB) have shown a lower microbiota diversity and produce higher amounts of BCFA in all intestinal segments compared to the other three pectins. Specific bacteria were differently affected by the supplemented pectins. In general in the caecum and colon, DM19 (HB), DM43 (HB) and DM49 (LB) pectins increased the relative abundance of Lachnospiraceae, *Lachnospiraceae NK4A136, Alloprevotella, Prevotellaceae UCG-001* and *Akkermansia,* while control and DM18 (LB) diets have shown a higher relative abundance of *Bifidobacterium*. The results of our research support the idea that very low amounts of pectin having specific structural features, such as more blocks of non-esterified GalA residues is able to affect microbiota diversity and composition and activate AhRs. Understanding the structure-function relationship between the gut microbiota and the specific structural properties of pectins is essential to predict their physiological effects.

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Appendix

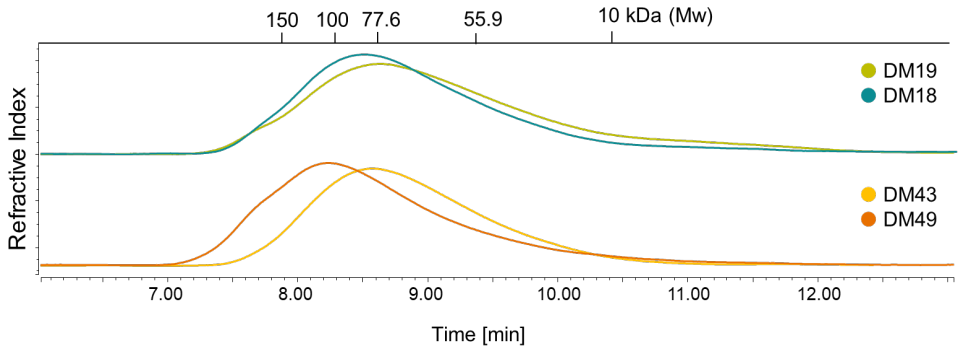

Figure S1. *HPSEC elution patterns of DM19, DM18, DM43 and DM49 pectins. Molecular weights of pectin standards (in kDa) are indicated.*

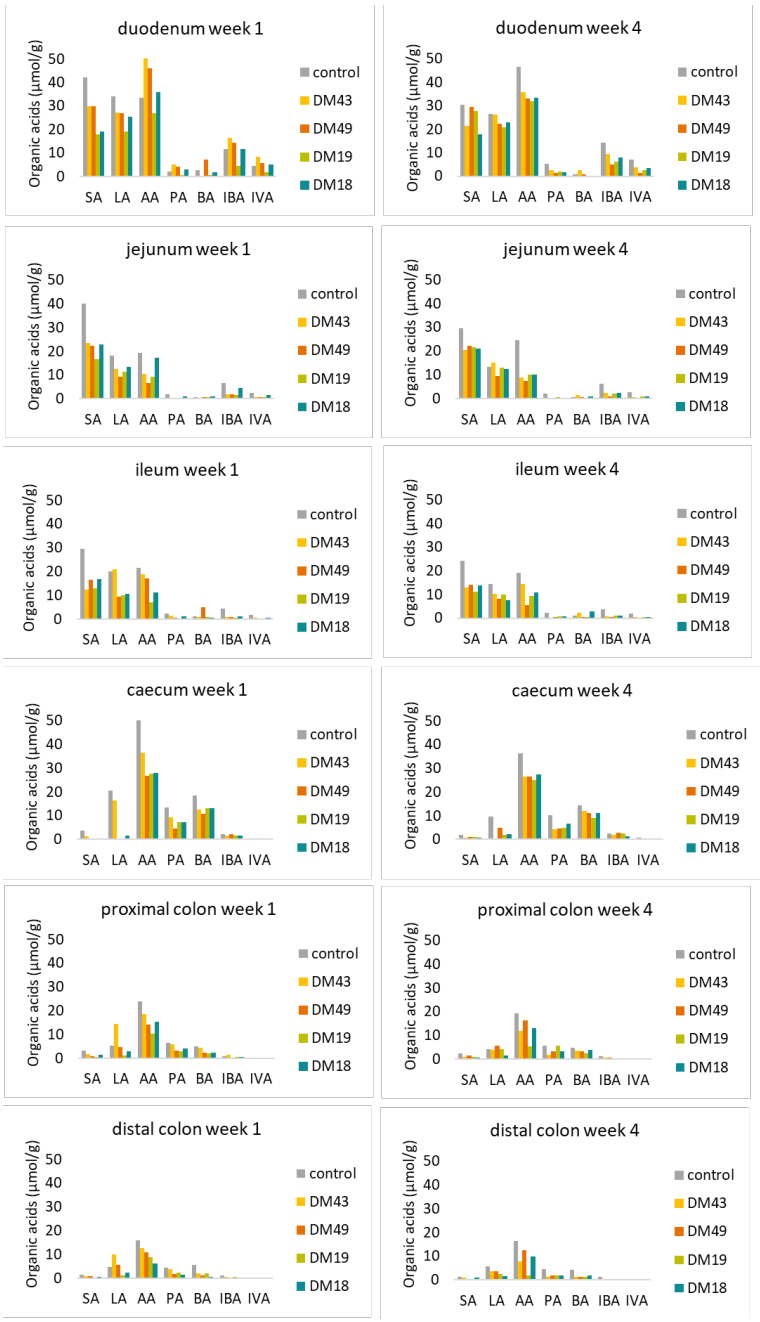

Figure S2. *Pooled digesta (n=10) organic acid concentrations from duodenum, jejunum, ileum, caecum, proximal- and distal colon in control mice and mice supplemented with DM18 (LB), DM19 (HB), DM43 (HB) and DM49 (LB) pectins. Samples were collected after 1 and 4 weeks. SA: succinic acid, LA: lactic acid, AA: acetic acid, PA: propionic acid, BA: butyric acid, IBA: iso-butyric acid, IVA: iso-valeric acid are shown in µmol/g dry matter of gut segment content.*

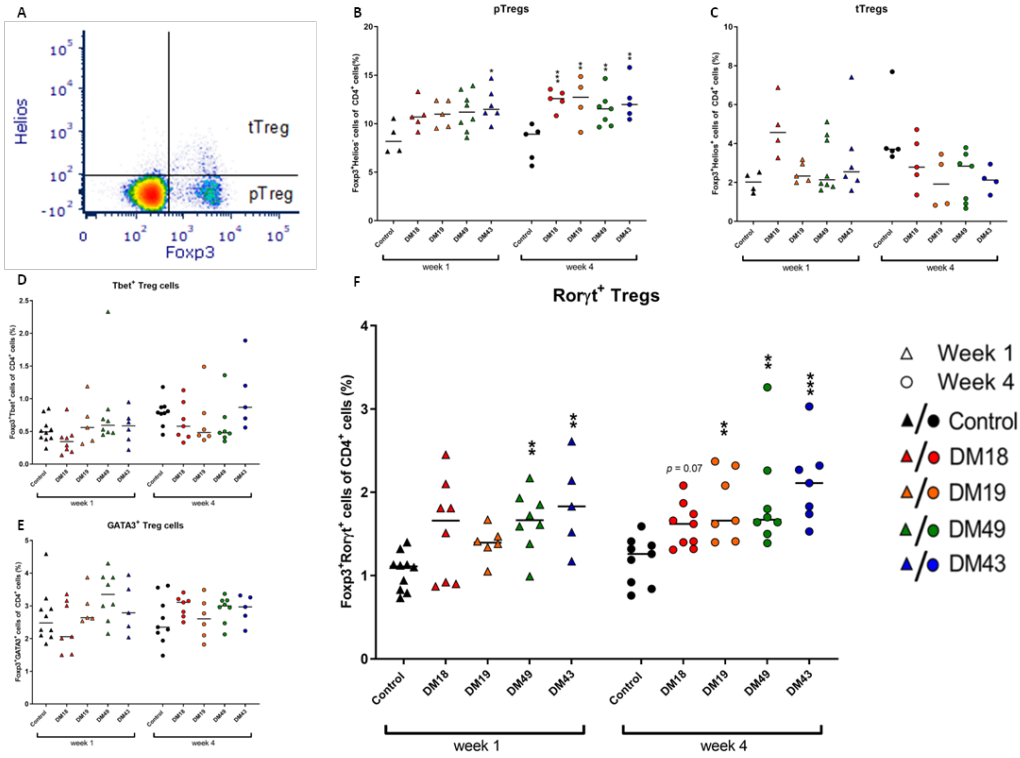

Figure S3. *Regulatory T cell frequencies of the mesenteric lymph nodes after 1 and 4 weeks of pectin supplementation. Peripheral induced T regulatory cells (pTreg) and Thymus derived T regulatory cells (tTreg) were selected by plotting Foxp3 and Helios from CD3+CD4+ T cells (A). Frequencies of pTregs (B), tTregs (C), Foxp3+Tbet+ Tregs (D), Foxp3+GATA3+ Tregs (E), and Rorγt+ Tregs (F) within CD4+ population in the MLN of control or pectin supplemented mice. Statistical significant differences between week 1 control and pectin treated mice or week 4 control and pectin treated mice are indicated by * (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001). Statistical differences between week 1 and week 4 within control or pectin groups are indicated by #. (Figure adapted from Beukema et al. [26])*

Chapter 6

General discussion

Research aim and approach

The development of pectin-based products requires knowledge of the structure-function relationships. A requirement for the elucidation of these structure-function relationships is the availability of well-defined pectins with known sugar composition, degree- and pattern of methyl-esterification and molecular weight. Knowledge of the structure-function relationship between pectins and health is limited. It is necessary to include well-characterized pectins in health studies to discover, explore and explain health benefits of such pectins. The aim of this PhD research was to use state-of-the-art analytical methods to fully characterize bioactive pectins by enzymatic fingerprinting techniques and to investigate their bioactivity through immune receptors and intermediate fermentation products in the intestinal tract of mice. The role of pectins in facilitating a healthy microbiota and their immunomodulatory properties were explored and correlated to their physico-chemical characteristics.

Fingerprinting methods and novel parameters were further developed to characterize and describe the methyl-ester distribution patterns of citrus pectins. Simultaneous endo-polygalacturonase (endo-PG) and pectin lyase (PL) digestion was used to degrade pectins sufficiently. This approach produced diagnostic oligomers to study the methyl-ester distribution patterns of parental and modified citrus pectins. The diagnostic oligomers were analyzed using High Performance Anion Exchange Chromatography (HPAEC) and subsequently Hydrophilic Interaction Liquid Chromatography Mass Spectrometry (HILIC-MS). This combined method enabled us to separate, identify and quantify the released oligomers and supported the development of descriptive parameters that help to define the methyl-esterification patterns of citrus pectins. Furthermore, the impact of the level- and distribution of methyl-esters on immune receptor signaling was determined. Since pectins have been shown to bind to Toll-like receptors (TLRs) in the small intestine and have direct effects on immunomodulation, molecular relations were measured both *in vivo* and *in vitro* and were simulated using molecular docking analysis. Eventually, the impact of structurally different citrus pectins on the gut microbiota and their fermentation metabolites were analyzed in mice.

Elucidation of the distribution patterns of methyl-esterified citrus pectins

It is difficult to relate a specific molecular structure of pectin to health-related properties. Various plant sources, extraction methods, different analytical methods, and the complexity of pectins make the characterization of pectins challenging. The increasing industrial, scientific, and public interest in bioactive and functional polysaccharides demands an advanced qualitative and quantitative investigation. Not only the level of methyl-esterification but also the methyl-ester distribution is essential in understanding pectin's specific techno- and bio-functionalities [1]. To understand the detailed structure of methyl-esterified pectins, homogalacturonan (HG) degrading enzymes have been applied to sufficiently degrade pectins into smaller diagnostic oligomers, followed by an exhaustive characterization by different chromatographic- and mass spectrometry methods [2, 3].

Simultaneous endo-PG and PL treatment can sufficiently degrade the pectin backbone, independently of the level and distribution of methyl-esters [2, 4] as demonstrated for methyl-esterified and acetylated sugar beet pectin. For this reason, in our study, the combined endo-PG and PL approach has been used to degrade the HG backbone of citrus pectins having varying levels of methyl-esterification into analyzable diagnostic oligosaccharides. As a result, it was possible to release a complex mixture of GalA oligomers which were both completely non-esterified and also partially methyl-esterified (**Chapter 2** & **Chapter 4**).

In previous research HILIC-ELSD-MS^n method was used for the analysis of acidic plant cell wall oligosaccharides, such as sugar beet pectin hydrolysates [5, 6]. However, the quantification of oligosaccharides using Evaporative Light Scattering Detection (ELSD) is not as reliable and reproducible as the quantification using HPAEC-PAD. In our study, HPAEC-PAD and HILIC-MS analysis were used for the quick identification and quantification of diagnostic oligomers after the enzymatic degradation. When using HPAEC, both the saturated and unsaturated GalA oligomers are well separated and can be easily quantified using available GalA standards, although information on the level of methyl-esters is lost because of the high pH-eluents. By analyzing the oligomers also with HILIC the methyl-esters are preserved, and the differently methyl-esterified oligosaccharides can be distinguished. Information on the saturated (non)methyl-esterified oligo GalA released by PG and the methyl-esterified unsaturated oligo galacturonides released by PL, can be used to characterize pectins in more detail. Peak annotation was done based on the *m/z* of the diagnostic oligosaccharides, and the relative abundance of selected DP (Degree of Polymerization) has been obtained after the integration of peak

areas in the chromatograms. The main advantage of using PG and PL together was that no polymeric pectin backbone segments remained un-analyzed, because the entire backbone was degraded into oligomers (**Chapter 2**). The separation, identification, and quantification of diagnostic oligomers using our method can be efficiently implemented for the analysis of pectic oligosaccharides (Figure 1).

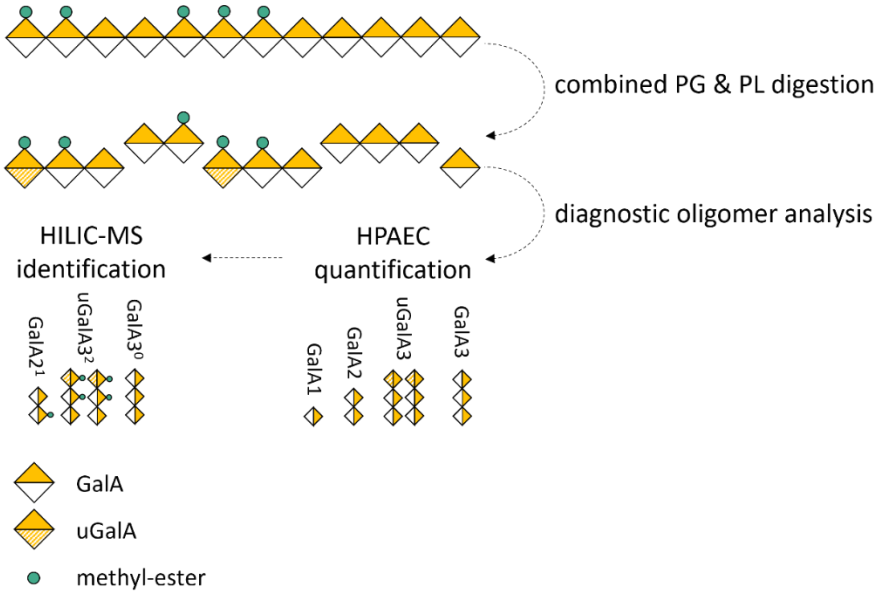

***Figure 1**. Schematic representation of the enzymatic fingerprinting approach to analyze the methyl-ester distribution of pectin polymers. GalA = Galacturonic acid, u = unsaturated GalA.*

Introducing the descriptive parameters

The descriptive parameters degree of blockiness (DB) and absolute degree of blockiness (DB_{abs}) are widely used and represent the number of non-esterified GalA present in blocks. However, DB and DB_{abs} do not differentiate between a limited number of large blocks or many small blocks [7].

Our fingerprinting method enabled the introduction of novel descriptive parameters to define the methyl-esterification patterns in citrus pectins. One of the new descriptive parameters that have been introduced is the degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme}) to represent the partly methyl-esterified GalA sequences. Therefore, DB_{PGme} can help to distinguish pectins with a similar DM, but different methyl-ester distribution patterns. DB, $DB_{abs,}$ and DB_{PGme} provide a view of the sequences of esterified

and non-esterified GalA residues of the pectin backbone where endo-PG is able to cut. In addition, using PL acting on the highly methyl-esterified GalA segments provides further information on the GalA segments which could not be degraded by endo-PG. Therefore, another novel parameter, the degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}) was introduced to distinguish the highly methyl-esterified segments in citrus pectins. The identification and quantification of the PG and PL degradable HG segments could discriminate both low and high DM pectins having similar DM values but different patterns of methyl-ester distribution (Figure 2) (**Chapter 2**).

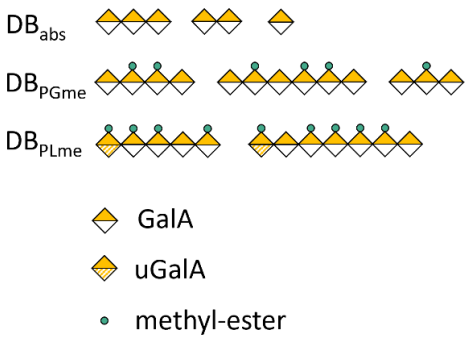

***Figure 2**. Schematic representation of pectic diagnostic oligomers released after enzymatic digestion with endo-PG of Kluyveromyces fragilis and PL. These diagnostic oligomers can be identified and quantified by HPAEC and HILIC and consequently the descriptive parameters DB/DB_{abs}, DB_{PGme} and DB_{PLme} can be calculated.*

DB and DB_{abs} indicate segments of non-esterified GalA residues over the pectin backbone [7, 8]. Another segment of the pectin is covered by DB_{PGme}, representing partly methyl-esterified GalA sequences, distributed in a random manner, since still PG-degradable. A third homogalacturonan structural segment is quantified by DB_{PLme} where a high DB_{PLme} value indicates sequences of highly methyl-esterified GalA residues, only degradable by PL.

The enzymatic fingerprinting using simultaneous endo-PG and PL digestion, and combined HPAEC-PAD and HILIC-MS analysis allowed the accurate characterization of the methyl-ester distribution patterns, which can be used to predict the techno- and bio-functionalities of citrus pectins. Enzymatic digestion of pectins followed by the analysis of diagnostic oligomers is an effective method to distinguish the pattern of methyl-esterification of different pectins with the help of descriptive parameters. DB_{abs}, DB_{PGme} and DB_{PLme} together reveal a diverse methyl-ester distribution pattern in rather

similar DM pectins and these parameters can be all obtained after the simultaneous digestion with PG and PL.

Moreover, the ratio of DB_{PGme}/DB_{abs} has been introduced (**Chapter 4**), which is the ratio of moderately methyl-esterified GalA oligomers represented by DB_{PGme} and the completely non-esterified GalA oligomers represented by DB_{abs}, both of which are quantified as PG released degradation products. The DB_{PGme}/DB_{abs} ratio indicates a distinct distribution pattern of the non-esterified GalA blocks over the backbone, where a higher ratio would indicate that the non-esterified blocks more closely associated with more dense methyl ester sequences. The ratio of DB_{PGme}/DB_{abs} reveals hidden patterns of the methyl-ester distribution.

Using our approach and parameters we were able to describe the entire pectin molecule. In contrast, Daas et al. have been using endo-PG alone to degrade the pectin, which only allowed the analysis of low DM pectins having more PG degradable segments and describing it with DB and DB_{abs} [9]. Analyzing only the low-esterified stretches of pectins via endo-PG hydrolysis gives just a partial view of methyl-ester distribution and will not enable judgement of the entire pectin [8, 9]. Our descriptive ratio DB_{PGme}/DB_{abs} provides the same information as the ratio of the non-esterified mono-, di- and triGalA to the (partially) methyl-esterified oligosaccharides released by PG as presented by Daas et al [8, 10]. We could not confirm the suggestion made by Daas et al [8, 10] that a higher amount of triGalA compared to mono- and tri GalA would illustrate longer PG degradable sequences. Another approach was used by Ralet et al. degrading high DM pectins using only PL, however, that could only degrade the very highly esterified segments of pectins, describing them using the parameters DBMe and $DB_{abs}Me$ [11]. Using PL, the highly methyl-esterified stretches can be studied, but it will also not fully elucidate the methyl-ester distribution over the entire homogalacturonan backbone, and the degree of degradation will still depend on the degree of methyl-esterification (DM), just like with PG alone [11]. The approaches or Ralet et al.[11] and Daas et al.[8] leave part of the pectin backbone unanalyzed.

Remoroza et al. have introduced the degree of hydrolysis by PG and PL, DH_{PG} and DH_{PL} respectively to elucidate the differences between sugar beet pectins [4]. The combined enzymatic approach has not been used before on citrus pectins, therefore we have been applying Remoroza's approach by using simultaneous PG and PL digestion to degrade the entire citrus pectin backbone during one incubation. Since sugar beet pectin derived oligomers still contain acetyl groups, the definition of the parameters were slightly re-defined in our study, although the principles of comparison are quite similar.

By using a combined enzymatic fingerprinting, the quantification of all the PG and PL degradable segments of pectin can discriminate both low and high DM pectins having different methyl-ester distribution patterns.

Descriptive parameters applied on pectins

For the present discussion 3 pairs of pectins were chosen, which were characterized using our methods and described with the new parameters (**Chapter 4**). The pairs are having similar DM and rather different descriptive parameters to show how much more information is hidden in the pectins which could not be revealed by DM or DB alone (Table 1). Using our descriptive parameters, rather big variations were observed between the very low DM pectins in spite of an 80% non-esterified backbone. Looking at their descriptive parameters it can be seen that L18 has a higher DB_{PGme} than L19, indicating a random pattern of methyl-ester distribution of that quite little number of methyl-esters for L18. As a confirmation, the DB_{PLme} was also somewhat different in the two pectins indicating a slightly higher blockiness for L19 pectin. Another surprise is the fairly big variation between the two commercial high DM pectins O64 and O59 coming from the same source and process. While the DB_{abs} of the O64 and O59 pectins are quite similar, DB_{PGme} and DB_{PLme} nicely represent the substantial differences possible in the methyl-esterified regions of the pectin backbone. The DB_{PGme} of O64 is 40% higher than of O59, meaning that there are many more randomly positioned methyl-esters in O59 which prevented PL to act as the methyl-esters are not right next to each other to match with the active site of the enzyme [2, 12]. The DB_{PLme} of O64 was found to be 18% higher than that of O59, suggesting that in O64 there were more methyl-esterified sequences close to each other. The re-esterified $O92_{R64}$ and $O85_{R59}$ pectins were similarly remarkable as L18 and L19 pectins. Even though their backbone is almost fully methyl-esterified the descriptive parameters could still reveal differences between the two. $O92_{R64}$ and $O85_{R59}$ pectins have low chances of having non-esterified GalA sequences, which is clearly shown by their rather low DB_{abs}. The very high DM is represented by the very high DB_{PLme} values. Yet, these very high DM pectins still had their own, slightly different, methyl ester distributions over their backbone also shown by their DB_{PGme} and the ratio of DB_{PGme}/DB_{abs}. Our aim was to create two similar fully esterified pectins, yet they both kept some of the properties of their parental pectins.

Table 1. *Descriptive parameters of commercial and modified pectins*

sample name	DM	DB_{abs}	DB_{PGme}	DB_{PLme}
L18	18	66	27	8
L19	19	75	21	10
O59	59	12	30	53
O64	64	14	18	65
O85 $_{RO59}$	83	2	14	99
O92 $_{RO64}$	92	3	10	75

Pectin conformation in relation to calcium and proteins

The primary structure of pectin mainly consists of the HG backbone interrupted with RG-I segments having neutral sugar side chains [19, 20]. Numerous properties of pectin in solution depend on the structure of polymers and on interactions with solvents, other polymer molecules and salts present [21]. Pectin's secondary structure and the conformation of the HG backbone are defined by the intermolecular orientation of GalA residues at the glycosidic linkages into a helical conformation [22]. This structure is a consequence of the reoccurring rotation angle of glycosidic linkages between the monomers [21, 22]. Higher level structures are formed by ionic interchain connections resulting in 3D structures such as gels or viscous solutions [23]. The total charge is defined by the DM and the charge density and charge distribution are defined by the DB and both contribute to the interactions and 3D conformation of pectins in solution [9, 24, 25]. Furthermore, the molecular structure, counter-ions, and pH are also essential parameters in pectin's conformation. The coil formation of pectin is also dependent on the blockwise or random distribution of methyl-esters over the HG backbone [17, 25]. Pectin helices are rather flexible which enables a high structural variability in solutions [21]. Polysaccharide helices can form networks with other molecules to generate stable conformations which greatly affects the molecule's function [21, 26]. In pectins, this will result in gelling via calcium or other divalent ions or even proteins [27-29]. In pectins calcium ions induce intermolecular associations, where the non-esterified GalA sequences, or junction zones are responsible for gel formation [15]. Pectin methyl-ester distribution patterns impact gelation and other functional properties of pectins [16]. Pectins with longer blocks of non-esterified GalA will have stronger and more efficient intermolecular interactions with other pectin molecules by calcium [17]. Nevertheless, even when identifying all parameters of pectins, it is still not easy

to know, neither to predict the functionality of a complex pectin mixtures, as with our analytical methods we are measuring average values of DM and DB/DB_{abs} rather than single molecule-specific information.

In **Chapters 3, 4 and 5** we described pectins with similar level of methyl-esters, but with a different methyl-ester distributions over the polymeric backbone, meaning that the charge over the backbone varied highly which could give rise to different conformations and interactions with ions, proteins and also receptor binding sites.

Below it will be described how the structurally different pectins affect immunity and the microbiota in mice. It was observed that two similar DM pectins showed different effects even though they were structurally rather similar. Therefore, it has been tested to see if there might be a difference in the gelling behavior of these two DM18 and DM19 pectins in different conditions in a quite simple experiment. Dietary fibers, such as pectins delay gastric emptying due to their viscosity, and gel formation [30]. Pectins were solubilized in ultra-pure water containing three different calcium concentrations: 1 mM calcium, 5 mM calcium and 10 mM calcium to a final concentration of 3 mg/ml pectin (3 mg/ml pectin concentration has been used also in the *in vivo* experiments described in **Chapter 5**). Upon mixing it was seen that the different calcium concentrations affected the gelling differently. As shown in Figure 3, 1 mM calcium hardly affected the gelling, while 5 mM calcium caused DM19 to form a rather solid gel and 10 mM calcium formed small gel lumps in the solutions instead of a gel (Figure 3.). For DM18 pectin none of the calcium conditions caused gelling.

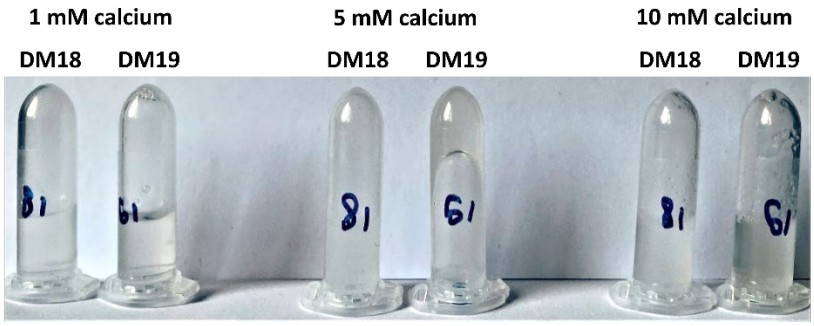

***Figure 3**. Gel formation of DM18 and DM19 pectin (3mg/ml) in 1, 5 or 10 mM calcium chloride solution.*

In low DM pectins the non-esterified sequences are believed to be long enough for egg-box formation. The formation of egg-boxes is only possible if

stretches of at least 8-12 subsequent non-esterified GalA residues are present [31, 32]. By this conformation large sequences of the pectin will be bound in junction zones, consequently, the chain length of the loose ends of the pectin molecules will decrease, causing reduced flexibility of the gel [33]. The ability to form egg-boxes does not exclusively depend on the level of methyl-esters, but also on the distribution patterns, therefore higher DB and DB_{abs} pectins with blocks of non-esterified segments will have more junction zones and a probability to form egg-boxes [28, 34]. Of the two pectins mentioned, DM19 pectin indeed was having a somewhat higher DB_{abs} (75, compared to 66 for the DM18 pectin) therefore it could be assumed that the methyl-ester distribution pattern of DM19 pectin was more advantageous in forming a stiffer gel, by having long non-esterified stretches.

Prior studies have also researched the influence of the DM and DB of pectins on proteins, namely the complex formation of β-lactoglobulin with pectin [29, 35]. It has been demonstrated that proteins and polysaccharides can form complexes due to electrostatic forces [36]. Varying DB, at equal DM play a key role in the complex formation of pectins with oppositely charged (bio)polymers like proteins. Low DM pectins in general have both a high overall charge and also a high local charge density, while high DM pectins having a high DB have a low overall charge, but may have a high local charge density. A randomly methyl esterified high DM, low DB pectin has both a low overall charge and a low local charge density [29, 35]. It was found that β-lactoglobulin favored binding to pectin regions having a high local charge density. From the DM and DB of pectins it seemed to be possible to predict the possibility of complex formation between pectins and proteins, but more information is needed [29]. The effects of the dimensions of a protein on the complex formation with pectins are substantial. Considering pectin's extended structure, there may be many possibilities for the proteins to bind, also depending on the properties of pectin used.

Linking pectin structure to bioactivity

Bioactive food ingredients have gained much attention lately from scientists, consumers, and food producers [37]. Natural bioactive foods are also called nutraceuticals, and they provide health benefits, such as the prevention or treatment of disease [38]. Such bioactive food ingredients have many modes of action. One mechanism by which dietary components may alter the immune actions is the interaction with receptors in the small intestinal epithelial cells to which e.g., polysaccharides can bind and activate or inhibit the activity of these receptors [39, 40]. Another mechanism is that they may interact with large

intestinal microbiota and change the microbiota composition, inducing physiological and immunological transformations in the body [41]. Consequently, an abundance of fermentation metabolites can be produced, such as short-chain fatty acids (SCFA), or amino-acid derivatives (indole derivatives) which may positively affect physiology [41-43]. To improve the beneficial use of bioactive food polysaccharides, next to an in-depth understanding of the mechanism, also a detailed characterization of the polysaccharide is essential. The aim of the research described in **Chapters 3, 4 and 5** was to gain understanding regarding both the direct and indirect effects of pectins specifically via the detailed characterization and binding to the TLRs.

Immunomodulation is affected by pectin structure

The small intestine is covered by a thin and loose mucus layer, which allows direct interactions of pectins with the underlying immune receptors in the intestinal epithelial cell layer [44, 45]. TLRs are one of the well-characterized pattern recognition receptors which are expressed in the small intestine. TLRs are able to recognize dietary fibers, such as pectins and consequently influence the underlying intestinal immune cells [46, 47]. However, the role of DM and DB of pectins in the influence of TLRs has not been analyzed in detail until now. Therefore, well characterized commercial and modified citrus pectins having different DM, DB_{abs}, DB_{PGme} and DB_{PLme} have been screened for TLR inhibiting abilities (**Chapter 3 & 4**).

Previously it has been shown that with lower DM in pectins TLR2/1 inhibition increased compared to higher DM pectins [39]. These findings suggested that a higher number of non-esterified GalA residues induce TLR2/1 inhibition. As low DM pectins are having long non-esterified blocks, it has been hypothesized that high DM pectins with a high degree of blockiness would possibly also have immunomodulating properties, therefore in the study described in **Chapter 3**, we aimed to confirm this hypothesis. The tested citrus pectins were mainly inhibiting TLRs, namely TLR2/1 dimerization was inhibited by the citrus pectins [40]. The impact of structural characteristics of the HG backbone of pectins on TLR2/1 inhibition is strongly dependent on the level and distribution of methyl-esters. The stronger inhibition of TLR2/1 by low DM (DM~20) pectins was confirmed. Furthermore, it was found that pectin with an intermediate DM and a high DB inhibited TLR2/1 just as strongly as very low DM pectins. The intermediate DM (DM~45) pectins having a low DB and two very high DM pectins, independently from their DB, both did not inhibit TLR2/1

effectively. This confirms that the influence of pectins to inhibit TLRs depends on structural characteristics.

Together, these findings showed that pectins having a blockwise distribution of non-esterified GalA sequences strongly inhibit TLR2/1 dimerization. The specific patterns of methyl-esterification of pectins may drive their affinity for binding and inhibiting TLR2/1 receptor. In **Chapter 3** it has been demonstrated that low and intermediate DM pectins having a high DB bind stronger to TLR2 compared to low DB pectins. Pectins with a high DB have more consecutive non-esterified GalA residues and by that higher charge density compared to low DB or random pectins. One of the prerequisites for the complex formation between proteins and pectins is the presence of favorable electrostatic interactions. Therefore, it could be hypothesized that the interactions of pectins with TLR2 are a result of electrostatic interactions. As high DB pectins specifically inhibited TLR2/1 dimerization, it has been presumed that certain pectin segments bind strongly to the TLR2 ligand-binding and dimerization regions [48]. Furthermore, in the study of Beukema et al. [49] a low DM pectin has been used to research the influence of calcium on pectin's TLR2 binding effect. In that study a stronger binding to TLR2 has been shown in low DM pectins in the absence of calcium. In contrast, when calcium was present, low DM pectin binding to TLR2 was weaker [49]. As calcium interaction happens with the negatively charged carboxyl groups, it is proposed that calcium-pectin interactions compete with the electrostatic interaction between pectins and TLR2.

In **Chapter 4** molecular docking analysis was described using two model molecules i.e., heptamer GalAs having two methyl-esters at distinct positions $GalA_7Me^{1,7}$ and $GalA_7Me^{2,5}$. Molecular relationships were analyzed and simulated. According to our findings at least 5 consequent non-methyl-esterified GalA residues are needed to bind to TLR2. Efficient binding may be strongly dependent on the 3D conformation of pectin, which will also influence the number of possible binding sites present. It is expected that when TLR2 has a pectin molecule bound to it, the large size of the pectin polymer (Mw ≈ 100kDa; ≈ 550-600 GalA residues) prevents the binding of the TLR2 agonist due to blocking the agonist binding site, and by that pectin will inhibit the dimerization with TLR1. Therefore, it is unlikely that pectin oligomers alone would inhibit TLR2/1 dimerization. It has been shown in another study, that in contrast to the specific pectin polymers, pectic oligomers do not activate TLR2 [46], which might be an indication that TLR2/1 dimerization would also not be inhibited by oligomers. The docking studies helped to recognize molecular interactions between pectin (oligomers) and TLR2 and may reveal why pectins with a specific structure bind to TLR2. In the

future it would be interesting to model longer oligomers with different methyl-esterification patterns, having longer and shorter non-esterified blocks and analyze their conformation and binding to the TLRs.

After confirming the pectin binding to TRL2 in a structure dependent manner, *in vitro* [39], our aim was to unravel pectin's immunomodulating effects in the intestine *in vivo* depending on the degree and distribution of methyl-esters.

Supplementation of pectin shapes microbial community

Fermentation of pectin and pectin's fermentation metabolites

A strong relationship between DM, DB_{abs}, DB_{PGme} and DB_{PLme}, and direct immune effects of pectins has been found. Therefore, it has been hypothesized that pectins may also indirectly influence immunity depending on DM and DB through the modulation of gut microbiota. The indirect effects of pectins were investigated to analyze how supplementation of structurally different pectins may influence microbiota and immune interactions in healthy mice. Recent studies have demonstrated that pectins can possibly modulate microbiota communities and influence intestinal immunity in a microbiota dependent way [50], however, it is not known how the structural characteristics of pectins impact the interaction between microbiota and immune cells. Therefore, in **Chapter 5** the impact of level and distribution of methyl-esters in pectins on the intestinal microbiota and immunity was studied after 1 and 4 weeks of supplementation with structurally different pectins in very low dose (3 mg/day) as a solution through oral gavage. The structure dependent effects of pectins on intestinal immunity were partly different than what was observed in **Chapter 3**. Previously we have shown *in vitro* that low DM (DM~20) pectins bind strongly to TLR2, however in the *in vivo* study from the two low DM pectins DM18 and DM19, only DM19 was shown to be immune active alongside with the intermediate DM pectins. Immune effects in the *in vivo* study have been measured through Aryl-hydrocarbon-receptor (AhR) activation. The activation of AhR by ligands has immune barrier protective effects [51] and also promotes a robust gastrointestinal immune barrier that is crucial to prevent pathogens from entering the bloodstream [52]. DM19 (HB), DM43 (HB), and DM49 (LB) pectins induced similar microbiota have also shown AhR activating effects which were different from the DM18 (LB) pectin fed mice. The AhR activation was present after both 1 and 4 weeks of supplementation. The three active pectins were different in their immune effects from the control and DM18 (LB) pectins, which may derive from the impact of these pectins on the intestinal microbiota composition. In the large intestine, DM19 (HB), DM43 (HB) and DM49 (LB) pectins induced a higher

relative abundance of *Lachnospiraceae, Lachnospiraceae NK4A136, Alloprevotella, Prevotellaceae UCG-001* and *Akkermansia,* while the control and DM18 (LB) supplemented mice have induced a higher relative abundance of *Bifidobacterium*. This suggests that the influence of pectins on the interaction between microbiota and immune cells may be dependent on specific pattern of methyl-esterification of pectins. Although the 1 and 4 weeks of pectin supplementation may give indications of the structure-dependent effects of these pectins on the microbiota and immunity. These findings suggest that the direct effects of pectins on TLR2/1 inhibition, as measured *in vitro*, may predict anti-inflammatory effects of pectins having specific methyl-esterification patterns *in vivo.*

Future murine studies using different doses and different pectin structures may give more suggestions on whether a given pectin structure is responsible for the observed effects. Moreover, both *in vitro* and *in vivo* fermentation studies including structurally different pectins might also identify specific microbial derived metabolites such as the AhR activating molecules with immune enhancing effects. Further research should be undertaken to investigate the differences between pectin supplementing methods. Dosing pectins in feed can be hard to control when mice get the feed *ad libitum*. In contrast, when feeding pectin as a solution through oral gavage the amounts can be controlled, but the 3D conformation of pectin may be different than when added in powder form mixed into the feed and may lead to different effects. The main difference between oral gavage and feed dosing of pectin is in pectin's viscosity and 3D conformation, therefore pectin may interact with the immune receptors and microbiota differently.

In our study, we have shown that very low doses of pectins, 1% of total fiber intake and 0.1% of the total feed intake for mice can already have positive effects on the immune system and microbiota. The very low dose of pectins may be inadequate to enhance SCFA production in the intestinal tract, but may be enough to induce the direct effects of pectins trough interactions with TLR2. In future investigations, it might be possible to use different doses of pectins to test what is the optimal dose for desired health effects. Moreover, the comparison of pectin supplementation in solution or mixed in the feed could be also investigated.

Modification of pectin structure

Tailoring of pectin's bioactivity

Pectin is one of the most important commercial polysaccharides, used in a wide range of food products [53-55]. In all products the fine structure of pectin is crucial in all its techno- and biofunctionality [39, 54, 56]. Commercial pectin production is based on only a few plant sources having the required properties. Citrus peel and apple pomace are the main sources of commercial pectin [55], their availability is also based on the huge volumes produced as side products. The strong, but not always known structure-function relationship has motivated this research to an improved understanding of the fine structure of pectin, with the goal of tailoring pectin's bioactivity. The aim would be to tailor and produce pectins with desired biofunctionality, which may be achieved by enzymatic and/or chemical modification of commercially extracted pectins from a wider range of raw materials using also different extraction processes.

Pectin modification already starts in the plant cell wall because a set of different pectin modifying enzymes is present to tailor the HG segments in particular and causing variations in the structure of the pectin [57, 58]. The enzyme mixture may be affected by the growing conditions or the ripeness of the plant and the stage of the harvest [59, 60]. These differences will result in complex mixtures of structurally different pectins when extracted [7]. Pectins present in the plant cell wall are usually highly methyl-esterified, however can be de-esterified while still in the plant cell wall by pectin methyl-esterases (PMEs) to achieve essential functionalities within the cell walls [57, 60]. The various plant PMEs have different mechanisms of action resulting in pectins with varying levels and distribution of the methyl-esters [61]. Pectins modified by the plant's own PMEs may change the plant cell wall by strengthening it (possibility of calcium gel formation) or weakening it, as the non-esterified GalA residues are ideal substrates for endo-PG action hence the pectin can be degraded *in muro* [58, 62] e.g. during the ripening process. Many of the pectic enzymes are not fully characterized yet. A thorough understanding of enzyme properties and modes of action could support the precise tailoring of pectins.

Commercial low DM pectins are commonly generated by controlled acid de-esterification of extracted HM pectin but also alkali or pectin methyl-esterase (PME) can be used [63]. Also, a more harsh acid extraction conditions will result in lower DM values of the final pectin, although also the molecular weight might become (much) lower. Where alkaline and acid conditions remove methyl-esters in a rather random fashion [9, 15]. Fungal and bacterial PMEs generally de-esterify the pectin backbone in a blockwise manner at pH 7-9 [2,

[61]. PMEs derived from fungal sources e.g., *Aspergillus aculeatus* de-esterify the HG backbone in a random way [2, 64]. However, some fungal PMEs e.g. the PME from *Trichoderma reesei,* may also act in a blockwise manner [65, 66]. Therefore, no straightforward prediction can be given on the precise methyl-ester distribution of the final modified pectin when using plant PMEs. Next to de-esterification, it is possible to fully methyl-esterify pectins by re-esterification using methanol acidified with either sulfuric or hydrochloric acid [67]. The re-esterification needs to be carried out at low temperatures (0-5 °C) to minimize the degradation of the pectin. The low temperatures also result in long reaction times, which even require 4 weeks, to acquire an almost fully methyl-esterified material.

We have created and analyzed both re-esterified and de-esterified pectins (**Chapter 4**). The methods and descriptive parameters were shown to be useful tools to differentiate both major and minor differences in the methyl-ester distribution patterns of pectins. It has been shown that pectins with similar DM and similar DB values highly differ from each other. An important finding was that by the modification of pectins, the *in vitro* TLR2/1 inhibition was also modified. In **Chapter 4**, comparing the parental pectins and their initial TLR2/1 inhibition with the re-esterified and subsequently alkali de-esterified pectins, it can be seen in two sets of pectins, that the inhibition decreased by randomizing the parental pectins. In the case of the O64 pectin by re- and de-esterification to DM55, the inhibition has dropped from 45% to 24%. For the O59 pectin, this difference was smaller, but in that case also the initial inhibition was lower. The present results show that, while the DM has dropped by 14%, the inhibition dropped by 47% caused by the rearrangement of methyl-esters. Our findings provide support for the conceptual premise that it is indeed possible to tailor the bioactivity of pectins and for that a detailed characterization is also necessary. Further research should be undertaken to tailor pectins using pure plant and/or fungal PME and create different sizes of non-esterified blocks over the pectin backbone. In the currently described research, it was possible to reveal fine structure of pectins, and by using alkali to de-esterify an almost fully esterified pectin we could show that indeed distribution patterns and non-esterified GalA blocks make a difference, but we could not yet tailor pectin to increase the TLR2/1 inhibition by testing different block sizes.

Relevance of our findings for further pectin characterization

A combined fingerprinting and analysis method was developed, alongside with novel descriptive parameters to enable the fast and accurate analysis of pectin oligomers. These methods and parameters enabled us to elucidate the structure of pectin polymers having different methyl-ester distribution patterns. There is a growing interest in pectin research, by which pectin is becoming increasingly important for various applications by the food- and pharmaceutical industries [68]. Information on pectin's ideal molecular structure can now be obtained and interactions with other molecules can be studied in a mechanistical manner. The possibility of tailoring the pectin structure enables the use of it also for new purposes. Such tailored pectins would have high added value and distinguished behavior which might spike the interest of both industries and research organizations.

The present research shows the value of such analytical methods not only for the characterization of pectins, but also for the development of new approaches to produce and characterize these functional pectins. Pectins are an example of multipurpose functional polysaccharides with many possible applications in a wide range of fields. Our method is a step forward in the characterization of pectins. The method could be more powerful if we could include standards for all GalA oligosaccharides above DP3 including also unsaturated GalA oligosaccharides or to establish the response factor of those saturated and unsaturated oligoGalAs. It is difficult to effectively separate mono- and oligosaccharides in one single run using chromatographic techniques. It has been proven that HILIC-MS is a valuable analytical tool for the identification of oligosaccharides [5]. However, this method is limited to the identification of diagnostic oligosaccharides up to DP 9. In the future novel chromatographic column materials may be able to further support our efforts of analyzing also higher DPs.

There are many different enzymes used for pectin characterization and these enzymes while having similar modes of action, may need different substrates to act. It is not appropriate to compare studies using endo-PG from different sources, i.e., *Kluyveromyces fragilis* or *Aspergillus niger*. In our study we have been using *K. fragilis* PG to degrade pectin, because it needs at least 4 adjacent non-esterified GalA residues to hydrolyze the backbone [9] and by that generating long enough oligomers and giving sufficient information on the methyl-esterification patterns. Some of the *Aspergillus niger* PGs can hydrolyze the backbone already between only two non-esterified GalAs, but by that generating too short GalA oligosaccharides for adequate analysis. We now have the tools to search for new and possibly more sensitive enzymes, for

example PGs needing more than 4 non-esterified GalA residues to split the pectin to release longer oligomers with even more information on the methyl-ester distribution.

In addition, since pectins in general are found to have a variety of different populations [13, 14], it is important to distinguish between intermolecular and intramolecular differences in methyl-esterification. This can be done by separating pectin mixtures by preparative chromatography based on size and/or charge [7].

Now we have the methods to study the biochemical properties of different enzymes and we will be able to select the appropriate enzymes to obtain a dedicated pectin for a specific purpose.

Consequences of our findings for the food/feed industry

In the food and feed industry pectins are commonly classified as high DM (DM > 50%) or low DM (DM ≤ 50%), with distinct techno-functionalities for the different industrial applications [55]. Our study provides perspectives on pectin characterization, with the goal to offer methods for future research for the targeted use of this highly versatile polysaccharide. More defined and known methyl-esterification patterns may promote different functionalities. In addition, a targeted modification or extraction process may make it possible to use pectins in novel applications by improved physico-chemical characteristics. By understanding pectin's molecular fine structure and technological effects, it may be used in new applications and the best functional pectins can be selected to improve the immune modulating properties and fermentation behavior of fibrous foods.

Moreover, since we do know now what type of methyl-ester distributions are required for the most effective TLR inhibition, we can now start to tailor pectins. Since a certain blockwise distribution is needed, the most ideal pectin may originate from a dedicated source, or plant PMEs may be used to come to the desired DB_{abs}, DB_{PGme}, DB_{PLme} values.

Consequences of our findings for the enzyme industry

Using the methods described in **Chapter 2** it is now possible to monitor and optimize pectin modification strategies. It is important to note that enzymes from various sources, and even enzymes from the same organism, are expected to have different substrate specificity, mode of action, bond-cleavage incidences, and substitution tolerances. Our method could be used

to determine the specific modes of action of different pectic enzymes. The mode of action of enzymes can be interpreted from the diagnostic oligomer profile obtained after enzyme action. Various genes from the genome of plant, fungi or bacteria, known or expected to be involved in the de-methyl-esterification of pectins could now be recognized and selected, corresponding enzymes expressed and studied in detail for their precise mode of action and substrate specificity.

Consequences of our findings for the pharmaceutical industry

Besides gaining understanding of the biofunctionality of pectins by enzymatic fingerprinting, these tools can also be used to characterize oligosaccharide mixtures to be used in pharmaceutical applications. The pharmaceutical applications of pectic oligosaccharides are very promising. Pectins have several pharmaceutical bioactivities, such as healing wounds, inducing apoptosis in cancer cells, preventing ulcers, or lowering cholesterol levels [37, 68-70]. Most of the studies present promising results of the beneficial effects of pectins and pectic oligosaccharides, however they mostly fail to describe mechanisms of action because of missing structural information [71]. To be able to design molecules for targeted effects, and predict the effects using our methodology could serve to better understand the structure-function relationships.

However, usually, there is quite a complex and poorly characterized mixture of pectic oligosaccharides used in studies. Our methods could vastly contribute to the revelation of the structure-function relationship of the pectic oligosaccharides by improving the identification and characterization of oligosaccharides e.g., DP or methyl-ester distribution patterns. The establishment of analytical methods to study the structure-function relationship and also the pharmacokinetics of pectic oligosaccharides is necessary to optimize pharmaceutical applications.

Conclusion

In conclusion, the analytical tools and parameters in this thesis may open new opportunities for the pectin analysis, which can contribute to the understanding of structure-function relationship of pectins and elucidating their fine structure.

The potential of pectins to interact and influence the immune receptors based on their chemical fine structure has been also clearly demonstrated. We have revealed the structure dependent fermentation of pectins, even at very low dietary fiber concentrations. Pectins having specific structural features were able to beneficially affect microbiota diversity and composition and have positive effects on the intestinal immune system through the activation of AhRs. Moreover, it was also shown that pectin fermentation and immune modulation behavior are strongly connected and should be studied together in the future to learn more on the intestinal health effects of pectins. It is now possible to tailor and characterize pectins for a healthy colon and a balanced immune system.

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Summary

Pectin is a plant cell wall polysaccharide, a profoundly abundant macromolecule found in higher plants. Pectin is applied widely in the food- and pharmaceutical industries. However, pectin's properties strongly depend on its chemical structure, which is not always predictable and persistent. Characterization of pectin in a rapid and detailed manner remains challenging due to its high diversity and complexity. The aim of this research project was to develop methods and extend the analytical toolbox to fully characterize the methyl-ester distribution patterns of pectins in detail to be able to explain or predict the bioactivities of pectin more accurately. Pectin's role in shaping the intestinal microbiota and the immunomodulatory properties were analyzed and linked to established physico-chemical characteristics.

The chemical features of pectins, as well as methods used to characterize their fine structure are described in **Chapter 1** of this thesis. Information is provided on the composition of native and commercial pectins including their techno- and biofunctionalities. In addition, approaches to determine the methyl-ester distribution of pectin using the complete enzymatic digestion of pectin into diagnostic oligomers are described. Various homogalacturonan degrading enzymes used for pectin depolymerization are discussed. Based on literature, existing analytical tools used for the characterization of pectins and pectin's various parameters are considered. Depending on their structural properties, pectins can influence the immune system directly or indirectly. Their beneficial effects through interactions with pattern recognition receptors and their influence on the intestinal immune system via the microbiota and fermentation metabolites are reviewed.

Chapter 2 focuses on citrus pectin analysis using an enzymatic fingerprinting method. Pectins were treated with endo-polygalacturonase (endo-PG) and pectin lyase (PL) simultaneously to reveal the methyl-ester distribution patterns over the homogalacturonan backbone. Hydrophilic interaction liquid chromatography (HILIC) with on-line electrospray ionization ion trap mass spectrometry (ESI-IT-MS) and high performance anion exchange chromatography (HPAEC) methods were optimized and validated to enable the complete separation, identification and quantification of the diagnostic oligomers released after the enzymatic treatment. Novel descriptive parameters were proposed to describe the methyl-ester distributions of pectin; i.e. degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme}) and degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}). Using these parameters it is possible to distinguish between three different segments; completely non-esterified,

partially methyl-esterified and very high methyl-esterified sequences of GalA residues. This method and parameters enabled us to clearly differentiate citrus pectins even with the same degree of methyl-esterification (DM). This approach is valuable to differentiate pectins and to be able to better predict their physical and biochemical functionalities.

In **Chapter 3** shows that pectins have anti-inflammatory properties by binding to Toll-like receptors (TLRs). Pectins can inhibit TLR2/1 dimerization in a DM-dependent manner as shown previously and now we demonstrate that the methyl-ester distribution of pectins has a highly relevant influence on TLR signaling as well. It has been shown that high DM, high DB pectins were able to bind to TLR2 and inhibit TLR2/1 dimerization almost as strongly as low DM citrus pectins. This demonstrates that not only the low level of methyl-esters, but also the blockwise distribution of non-esterified GalA residues, is responsible for the TLR2/1 inhibition. With this knowledge, it is possible to gain a better understanding of the structural characteristics of pectins having TLR2/1 inhibiting properties. Pectins with special structural properties can now be designed for novel functional food applications.

To better understand the underlying mechanisms involved in pectin-TLR2 binding, in **Chapter 4** the relationship between pectin methyl-ester distribution patterns and conformation, and the inhibition of TLR2/1 dimerization is described. Pectins were extensively characterized using the methods, and descriptive parameters as mentioned in Chapter 2 and the structural mechanisms underlying the inhibition of TLR2/1 dimerization were studied. Furthermore, docking simulations were performed, and the molecular relations between pectin and TLR2/1 were measured using two GalA heptamers having methyl-esters on different positions. The GalA heptamer which had five consequent non-esterified GalA residues has shown better fitting to the TLR2, than the heptamer with only two free GalAs next to each other. The detailed structural analysis of 6 pairs of pectins having similar DM, but different DB demonstrated that interactions with TLR2 are occurring in a structure-dependent way. It has been found that a blockwise pattern of methyl-esterification is needed for the strongest inhibition where the non-esterified blocks are not too long, but also not too short. It has been demonstrated that the ratio of partially methyl-esterified to non-esterified oligomers released by PG (DB_{PGme}/DB_{abs}) does point to the patterns of methyl-esterification. These results further corroborate the understanding of the molecular interactions between pectins and TLRs. This knowledge may be used in the future to tailor pectins for the prevention of inflammation.

Chapter 5 provides information on the fermentability and immune effects of pectins. The effect of structurally different pectins on murine gut microbiota and fermentation was investigated. The effect of pectins that differ in DM and DB_{abs} on the microbiota composition and fermentation products was studied in different parts of the mouse intestine i.e., the duodenum, jejunum, ileum, caecum, proximal- and distal colon. Three mg of structurally different pectins, counting for 1% of the total daily fiber intake of mice, were supplemented as solution through oral gavage to healthy mice. Intestinal samples were collected on day 7 and day 28. It was found that intermediate DM pectins and a very low DM with a high DB pectin effectively increased microbiota diversity, especially in the large intestine. In the caecum, an increased relative abundance of Lachnospiraceae, *Lachnospiraceae NK4A136, Alloprevotella, Prevotellaceae UCG-001* and *Akkermansia* was found. The control and the low DB low DM pectin supplemented diets induced the relative abundance of *Bifidobacterium*. Fermentation metabolites, such as the SCFAs found in the intestinal segments of the mice were mostly unaffected by the different diets or decreased compared to the control diet. Other metabolites, such as Aryl-hydrocarbon receptor (AhR) activating components were also measured, and it was found that pectins with a specific structure activate AhRs. Our results demonstrate that pectins with specific structural features even in a very low amount are able to positively affect microbiota diversity and composition and have beneficial effects on the intestinal immune system through the activation of AhRs. Knowing structure-function relationships between the intestinal microbiota and the pectin structure helps to predict beneficial physiological effects of pectin.

In the final **Chapter 6**, the impact of the main findings of this PhD thesis was discussed and reflected upon. Main considerations of the fingerprinting methods and descriptive parameters used to characterize pectins in fine detail are presented. The structural characteristics of commercial and modified citrus pectins through the descriptive parameters were addressed. The implications of the health effects of pectins depending on their methyl-ester distribution patterns are also discussed while taking into account the limitations of some of our methods and the possibility to tailor pectins for the best functionality. Besides, the in-depth characterization of intact citrus pectins by enzymatic fingerprinting and the novel analytical method developed are very useful and can in the future be used to explain a wide range of applications of pectins.

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Köszönet a családomnak és barátaimnak!

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És végül de legkevésbe és sem utolsó sorban szeretném megköszönni a családomnak, elsősorban, hogy vagytok nekem, hogy mindig támogattok és felvidítotok, és hogy veletek nem unalmas az élet. Szeretlek benneteket! Köszönöm **Mimu**, **Czirmók**, **Ádibogyó** és **Mimó-Adél** a sok szeretetet és örömöt amit kaptam, sokszor baba fotók formájában is, sok erőt adott nekem! Köszönöm **Adri**, hogy helyettem is gondoskodsz a megjelenésemről, és hogy a kreativitásod nem csak külsőségekre korlátozódik. **Anna**, köszi, hogy ilyen jófej kis csajjá nőttél időközben! **Ágnes**, köszi, hogy gondoskodol apáról és **Csaba**, köszi, hogy vigyázol az anyucira! Köszönöm **Anyuci** és **Apuci**, és **Mamzi** hogy kemény munkával minden anyagi, lelki és szellemi dolgot megadtatok nekünk, amire valaha szükségünk lehet. És legjobb barátom és társam, köszönöm **Zsolti** a támogatást és, hogy kitartottál mellettem, biztosítottad a sétáinkat, futásainakt és mesés helyekre vittél kirándulni!

With that, I hope to have mentioned as many people as my memory allows me. Please forgive me if I have not mentioned you and remember that you have been incredibly important in my journey!

♡ Csibe / Éva

About the author

Curriculum Vitae

Éva Jermendi was born on September 27th 1988 in Pécs Hungary. In 2007 she was admitted at the University of Pécs, Hungary where she studied Dietetics. In 2010 she obtained a scholarship within the Erasmus exchange program to spend a semester at the Hogeschool van Arnhem en Nijmegen (HAN), the Netherlands, to focus on nutrition and evidence-based science. She finalized her BSc studies with a thesis entitled "The efficacy of chemoprevention based on molecular epidemiological investigation of chalcone-containing mixtures" for which she received the Dean's prize for the best thesis in 2011. After her BSc studies, she worked as a registered dietitian at the Zsigmondy Vilmos Hospital of Harkány, Hungary. In 2013 she continued her studies with an MSc in Nutrition and Health with a specialization in Molecular Nutrition and Toxicology at Wageningen University. Her thesis focused on the effect of quercetin conjugation on protein kinase signaling pathways in HepG2 cells, which was performed at the Division of Toxicology, WUR. As part of her MSc program, she conducted a research internship at TNO, Zeist, the Netherlands, where she worked on two projects, the germ-free cultivation of zebrafish embryos to examine potential differences in locomotor activity and histopathology; and the quantification of the uptake and biodistribution of test compounds in zebrafish embryos and larvae. In 2015, she obtained her MSc degree in Nutrition and Health and in 2016 she started as a PhD candidate at the Laboratory of Food Chemistry under the supervision of Prof. Dr. Henk A. Schols. This research was performed in the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center. The results of her PhD research are presented in this thesis. Éva continued to work at the Carbohydrate Competence Center as a project manager.

Contact: jermendi.eva@gmail.com

List of publications

É Jermendi, M Beukema, C Fernández-Lainez, P de Vos, HA Schols, The influence of pectin methyl-esterification patterns on TLR 2/1 inhibition, *Submitted for publication*

É Jermendi, M Beukema, MJ Logtenberg, R An, EG Zoetendal, P de Vos, HA Schols, Structure dependent influence of low doses of pectins on the gut microbiota composition of mice, *Submitted for publication*

D Liu, J Bi, X Liu, J Liu, **É Jermendi**, HA Schols, A wide diversity exists in pectin structure from thirteen apple cultivars, *Submitted for publication*

M Beukema, **É Jermendi**, MP Oerlemans, MJ Logtenberg, R Akkerman, R An, MA van den Berg, EG Zoetendal, T Koster, C Kong, MM Faas, HA Schols, P de Vos, The level and distribution of methyl-esters determine the impact of pectin on intestinal T cell immunity, microbiota composition, short-chain fatty acid production and aryl-hydrocarbon receptor activation in healthy mice, *Carbohydrate Polymers,* 2022, 286, 119280.

É Jermendi, M Beukema, MA van den Berg, P de Vos, HA Schols, Revealing methyl-esterification patterns of pectins by enzymatic fingerprinting: Beyond the degree of blockiness, *Carbohydrate Polymers*, 2021, 118813

M Beukema, R Akkerman, **É Jermendi**, T Koster, A Laskewitz, HA Schols, MM Faas, P de Vos, Anti-inflammatory properties of pectins in mice with *C. rodentium* - induced colitis, *Molecular nutrition & food research*, 2021, 65 (19), 2100346

M Beukema, **É Jermendi**, T Koster, K Kitaguchi, BJ de Haan, MA van den Berg, MM Faas, HA Schols, P de Vos, Attenuation of Doxorubicin-Induced Small Intestinal Mucositis by Pectins is Dependent on Pectin's Methyl-Ester Number and Distribution, *Molecular nutrition & food research*, 2021, 65 (18), 2100222

M Beukema, **É Jermendi**, MA van den Berg, MM Faas, HA Schols, P de Vos, The impact of the level and distribution of methyl-esters of pectins on TLR2-1 dependent anti-inflammatory responses, *Carbohydrate Polymers*, 2021, 251, 117093

M Beukema, **É Jermendi**, HA Schols, P de Vos, The influence of calcium on pectin's impact on TLR2 signalling, *Food & Function*, 2020, 11, 7427-7432

SBR Prado, M Beukema, **É Jermendi**, HA Schols, P de Vos, JP Fabi, Pectin interaction with immune receptors is modulated by ripening process in papayas, *Scientific reports*, 2020, 10 (1), 1-11

Overview of completed training activities

Discipline specific activities

Courses

Summer course Glycosciences [a]	VLAG, Groningen, 2016
Advanced Food Analysis [a]	VLAG, Wageningen, 2017
Food Enzymology and Biorefinery [a]	VLAG, Wageningen, 2017

Conferences and meetings

The 5th beneficial microbes conference [a]	Bastiaanse Communication, Amsterdam, 2016
The 7th International Dietary Fibre Conference	ICC, Rotterdam, 2018
The 6th European Polysaccharide Network of Excellence (EPNOE) Conference [a]	EPNOE, Aveiro, Portugal 2019
The 20th Gums and Stabilisers for the Food Industry Conference [a,b]	Food Hydrocolloids Trust, San Sebastian, Spain, 2019
CCC symposium [a,b]	CCC, Groningen, 2016-2020

General courses

VLAG PhD introduction week	VLAG, Baarlo, 2016
Brain training	WGS, Wageningen, 2016
Competence assessment	WGS, Wageningen, 2016
Interpersonal communication for PhD candidates	WGS, Wageningen, 2016
Effective behaviour in your professional surroundings	WGS, Wageningen, 2017
Scientific Writing	WUR in'to Languages, Wageningen, 2019

Optional courses and activities

Preparation research proposal	FCH, Wageningen, 2016
PhD study trip 2016 [a,b]	FCH, Japan, 2016
PhD study trip 2018 [a,b,c]	FCH, Austria – Italy, 2018
BSc/MSc thesis students supervision, presentations and colloquia	FCH, Wageningen, 2016-2020
PhD presentations	FCH, Wageningen, 2016-2020
Project meetings	CCC Consortium, 2016-2020

[a] Poster presentation. [b] Oral presentation. [c] Organizing committee
Abbreviations: VLAG, Graduate School for Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Science; CCC: Carbohydrate Competence Centre; WGS: Wageningen Graduate Schools; FCH: Laboratory of Food Chemistry, ICC: International Association for Cereal Science and Technology.

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