The level and distribution of methyl-esters influence the impact of pectin on intestinal T cells, microbiota, and Ahr activation

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ABSTRACT

Pectins are dietary fibres that modulate T cell immunity, microbiota composition, and fermentation profiles, but how this is influenced by the degree of methyl-esterification (DM) and degree-of-blockiness (DB) of pectin is unknown. Here, we demonstrate that supplementation of DM19(high-DB), DM49(low-DB) and DM43(high-DB) pectins at a low dose increased the frequencies of intestinal T-helper (Th)1 and Th2 cells after 1 week of pectin supplementation in mice, whereas DM18(low-DB) did not. After 4 weeks of supplementation with those pectins, Th1 and Th2 frequencies returned to control levels, whereas Rorγt[+] regulatory T-cell frequencies increased. These structure-dependent effects could derive from induced shifts in microbiota composition that differed between DM18(low-DB) pectin and the other pectins. T-cell-modulating effects were not short-chain-fatty acid-dependent, but rather through an increase in Aryl-hydrocarbon-receptor-activating components. Thus, pectins with a specific combination of DM and DB have an impact on intestinal T cell-immunity in mice, when supplemented at a low dose.

1. Introduction

Low dietary fibre intake in the western industrialized countries has been linked to an increased prevalence of immune-related disorders, such as inflammatory bowel disease, allergies and autoimmune disorders (Berer et al., 2018; Maki & Phillips, 2015; Oliveira et al., 2013; Sonnenburg & Sonnenburg, 2014; Van Itallie, 1978). These diseases occur at very low frequencies in more traditional societies that consume higher fibre diets (Burkitt, Walker, & Painter, 1972; Sonnenburg & Sonnenburg, 2014). The exact mechanisms explaining how dietary fibres prevent the development of these immune-related disorders are not fully understood. It is believed that a low dietary fibre diets alters gut

microbiota composition and its metabolism that consequently disturbs host-microbiota interactions (Sonnenburg & Sonnenburg, 2014). Sufficient intake of dietary fibre can however influence the intestinal microbiota composition, which in turn may beneficially stimulate intestinal immunity (Makki, Deehan, Walter, & Bäckhed, 2018).

Recent research showed that the intestinal microbiota modulates T cell responses that play a central role in intestinal immunity (Pezoldt, Yang, Zou, & Huehn, 2018). T cell immunity can be influenced by the intestinal microbiota after recognition of microbiota derived-antigens that are presented by antigen presenting cells (Pezoldt et al., 2018) or through the secretion of microbial metabolites, such as short-chain fatty acids (SCFAs) (Smith et al., 2013) or tryptophan metabolites (Ye et al.,

2017). In particular, the intestinal microbiota can activate effector cells which are defined by the transcription factors T-bet (T helper 1; Th1), GATA3 (Th2), and RORγt (Th17) (Eberl, 2016). These effector cells protect against microbial threats, but excessive responses of these cells may also lead to the development of inflammatory or autoimmune diseases, or allergy (Josefowicz, Lu, & Rudensky, 2012; Van Wijk & Cheroutre, 2010). To maintain intestinal homeostasis, Foxp3 expressing regulatory T cells (Tregs) balance these exacerbated immune responses (Josefowicz et al., 2012). The intestinal microbiota may, therefore, be an effective target to influence T cell immunity and prevent the development of immune-related disorders (Siracusa, Schaltenberg, Villablanca, Huber, & Gagliani, 2019).

Pectin is a dietary fibre molecule that has been demonstrated to modulate T cell responses in a microbiota-dependent manner (Bernard et al., 2015; Wu et al., 2019). Beneficial effects of pectins depend on its chemical structure (Chen et al., 2006; Ishisono, Yabe, & Kitaguchi, 2017; Popov et al., 2013; Sahasrabudhe et al., 2018; Sun et al., 2017; Vogt et al., 2016). Commercial pectins consist mainly ($\geq$ 70%) of linear 1,4-D-galacturonic acid (GalA) (homogalacturonan) segments and branched rhamnogalacturonan segments (Caffall & Mohnen, 2009). The homogalacturonan backbone can be methyl-esterified (Supplementary Fig. 1), and the percentage of methyl-esterified GalA residues on the pectin is determined by the degree of methyl-esterification (DM) (Thakur et al., 1997). These methyl-esters can also be differently distributed over the pectin backbone, which is determining the degree of blockiness (DB). The DB is a structural parameter for the distribution of non-esterified GalA residues in a pectin. Pectins with a high DB have a more block-wise distribution of non-esterified GalA residues, whereas pectins with a low DB have a more random distribution of non-esterified GalA residues (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999).

Pectins have anti-inflammatory effects on immunity by direct effects on Toll-Like-Receptor (TLR) 2–1 signaling or by supporting production of beneficial microbial products by gut microbiota. The extent to which pectins have these beneficial effects seem to be dependent on the structural characteristics of pectin such as the DM and DB (Beukema et al., 2021; Beukema et al., 2021; Sahasrabudhe et al., 2018). Sahasrabudhe et al., 2018 demonstrated in vitro that pectins with a low DM inhibit TLR2–1 signaling stronger than pectins with a high DM (Sahasrabudhe et al., 2018). Moreover, our group also showed in TLR2 expressing cell-lines that high DM pectins combined with a high DB can still have TLR2–1 inhibiting capacity despite its high DM (Beukema, Jermendi, Koster, et al., 2021). Additionally, the DM does also influence the utilization of pectin by microbiota and the production of metabolic products as pectins with a different DM induce different effects (Dongowski, Lorenz, & Proll, 2002; Larsen et al., 2019; Tian et al., 2016; Tian et al., 2016).

Pectins can modulate microbiota composition, microbial fermentation, and T cell responses (Bernard et al., 2015; Wu et al., 2019), but to what extent this is influenced by DM and DB is unknown. We hypothesized that pectin influences T cell immunity in a DM and DB dependent manner through modulation of the intestinal microbiota. To investigate this, healthy mice were supplemented with pectins which differ in DM and DB at a dose of 3 mg/day. Mice were supplemented for 1–4 weeks to determine the short and long term effects of pectin supplementation. We determined the impact on intestinal and systemic T cell frequencies, microbiota composition, and fermentation profiles of four structurally different pectins after administration in mice: two low DM pectins and two high DM pectins and within each DM-group a low DB pectin and a high DB pectin.

2. Material and method

2.1. Pectins

Commercially extracted pectins from lemons (CP kelco, Copenhagen, Denmark) with DM of 18, 19, 43, and 49% were used in the current study. Molecular weight, monosaccharide content, the DM, and the DB were determined as previously described (Beukema, Jermendi, Koster, et al., 2021).

2.2. Mice

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). All mice were acclimatized for 1.5 weeks before the start of the experiment. Animals were cohoused with a total number of 4 mice in individual ventilated cages. Mice were given ad libitum access to sterilized water from the tap and RMH-B food (AB Diets, Woerden, The Netherlands) (Sahasrabudhe et al., 2018). The basal of level of pectin in the diet was minimal (Tian et al., 2019).

After 1.5 weeks of acclimatization in our facility, mice were fed with pectins for 1 or 4 weeks. Pectins were administered twice a day through oral gavage in a volume of 250 μL per gavage (6 mg/mL). Oral administration of this pectin dose was previously determined to induce minimal discomfort to animals (Sahasrabudhe et al., 2018). Control mice received 250 μL of sterile water. Each experimental group contained 8 mice. Mice were anesthetized using isoflurane/O$_2$ and sacrificed by cervical dislocation. Digesta from caecum was collected for the determination of microbiota composition, fermentation profiles and Aryl hydrocarbon receptor (Ahr) activating components. Spleen was collected as representative of systemic immunity (Bronte & Pittet, 2013) and mesenteric lymph nodes (MLN) were also collected to study the impact of pectin supplementation on intestinal immunity (Spahn et al., 2002).

2.3. Isolation of immune cells and flow cytometry of T cells

Cells were isolated from the spleen or MLN for immune cell staining as previously described (Faas et al., 2020). To stain T cell subsets, 1×10^6 spleen or MLN cells were transferred to a 96 well plate and centrifuged at 600 xg for 5 min. at 4 °C. The cells were washed with PBS (Lonza, Basel, Switzerland) and incubated for 15 min. with ZombieNIR (Supplementary Tables 1 and 2). Next the cells were washed with FACS buffer (PBS + 2% dFCS (Sigma Aldrich)) and incubated for 10 min. with 50 μL extracellular blocking buffer (20% (v/v) rat serum (Jackson, Newmarket, UK), 78% (v/v) FACS buffer and 2% (v/v) FC block (eBioscience, Vienna, Austria) to prevent non-specific binding of antibodies. This was followed by an incubation of 30 min. with 25 μL extracellular antibody mix (Supplementary Tables 1 and 2). Then the cell suspension was washed with FACS buffer and fixed for 30 min. in FACS lysing buffer (BD Biosciences, Breda, the Netherlands). Next, the cells were washed twice with permeabilization buffer (Demi water +5% (v/v) PERM (Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA). Then the cells were intracellularly blocked for 10 min. with intracellular blocking buffer (normal rat serum (20% (v/v) in permeabilization buffer). After this step, the cells were incubated for 30 min. with 50 μL intracellular antibody mix (Supplementary Tables 1 and 2). Then, the cells were washed twice with permeabilization buffer. Finally, the cells were resuspended in 100 μL FACS buffer and stored at 4 °C until analysis within 16 h. Washing steps included centrifugation at 600 xg for 5 min. at 4 °C. FMO controls were used to set the gates (Supplementary Fig. 2).

2.4. Flow cytometry of T cells

FACSverse flow cytometer system was used to analyze samples (BD Biosciences Franklin Lakes, USA), using the FACSsuite software. Analysis was performed by FCS Express software version 6 (De Novo Software, Pasadena, USA).

2.5. Ahr activation assays

To study whether caecal digesta samples contained Aryl hydrocarbon receptor (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. The Lucia luciferase reporter gene is placed under the control of an Ahr-Ahr nuclear transporter responsive promotor. Upon activation of the Ahr by Ahr-activating components, high levels of intracellular Ahr will dimerize with Ahr nuclear translocator which will lead to the secretion of the Lucia luciferase reporter protein which can be quantified by LuciaGold substrate (Invivogen). Ahr-expressing cells were cultured in DMEM culture media (Lonza) containing 10% dFCS, 50 U/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 μg/mL Zeocin (Invivogen).

Cells were seeded at a density of 50.000 cells/well in a 96 well plate (Costar) and incubated for 24 h. 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 negative control. After 24 h 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.

2.6. Microbiota analysis

Mice digests from caecum were collected from 200 animals, and immediately stored at −80 °C. Microbiota composition was determined by sequencing barcoded 16S ribosomal RNA (rRNA) gene amplicons while using Illumina Hiseq2500 (2 × 150 bp). DNA was isolated using Repeated-Bead-Beating (Salonen et al., 2010) 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 isolated DNA as 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, The Netherlands), and quantified using the QubitTM dsDNA BR Assay kit (Invitrogen). An equimolar mix of purified PCR products was prepared and sent for sequencing (GATC-Biotech, Konstanz, Germany, now part of Eurofins Genomics Germany GmbH). Raw sequence reads were subsequently processed while using NG-Tax 2.0 (Poncheewin et al., 2020) with default settings and R version 4.0.3. Amplicon sequence variants (ASVs) with less than 0.1% relative abundance were removed. The taxonomic assignment was performed with a threshold of 80% using the SILVA reference database release 132 (Quast et al., 2012). Relative abundances of bacteria at genus and phylum level were calculated using the functions *tax_glom* and *transform* in the *phyloseq* (McMurdie & Holmes, 2013) and *microbiome* (Lahti & Shetty, 2017) R package respectively.

2.7. Organic acids profiling

SCFAs were measured in the pooled mice digests from caecum. Between 20 and 150 mg pooled digesta was dissolved in 200 μL nuclease free water, mixed, and consequently centrifuged (20,000 ×*g* for 10 min. on 4 °C). After mixing and centrifugation, 100 μL supernatant was transferred to vials and used for analysis. SCFAs were quantified using a Dionex Ultimate 3000 HPLC (Thermo Scientific, Dionex, Sunnyvale, CA, USA). 10 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 (Ladirat et al., 2014). Standard solutions of lactic acid, succinic acid, acetic acid, propionic acid, and butyric acid were prepared in concentrations of 0.05–3 mg/mL. Data were processed using Chromeleon 7.2 (Thermo Scientific). SCFA concentrations were expressed in μmol mg^{-1} dry matter to correct for the potential impact of digest consistency. Dry matter content was determined by drying the samples in an oven overnight at 60 °C.

2.8. Statistics

Statistical analysis on results was performed using Graphpad Prism program (La Jolla, CA, USA). Normal distribution of immune and microbial metabolite 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 (Statistical differences were indicated with *). Post-testing between week 1 and week 4 was performed with Sidak post-test (Statistical differences were indicated with #). Significant correlation between the relative abundance of genera and the mean of Th1, Th2, or Rorγt⁺ Treg levels was determined with Spearman's rank correlation test. Correlation was considered significant when the absolute value of Spearman's rank correlation coefficient (Spearman's r) was >0.6 and statistically significant when $p <$ 0.05. *P*-values of correlation data were corrected for multiple testing by the false discovery rate (FDR) of Benjamini–Hochberg (FDR < 0.05).

3. Results

3.1. Characterization of pectin

Four pectins that have been demonstrated to induce DM and DB dependent effects on TLR2 signaling were used in the current study (Beukema, Jermendi, van den Berg, et al., 2021). The degree (percent) of methyl-esterification (DM), degree of blockiness (DB), molecular weight and sugar composition was previously characterized for these pectins (Table 1; (Beukema, Jermendi, van den Berg, et al., 2021)). The pectins were homogalacturonan pectins that showed minor differences in sugar composition. The pectins did however differ in DM or DB. The pectins can be grouped into two levels of similar DM of ≈19% (DM18 and DM19) and ≈46% (DM43 and DM49). For each DM group, there was a pectin with a lower DB (DM18, DM49) and a pectin with a higher DB (DM19 and DM43). Lower DB pectins with DM18 and DM49 had a respective DB of 86 and 33, and higher DB pectins with DM19 and DM43 had a DB of 94 and 60, respectively. As low DM pectins have a high number of non-esterified GalA residues, the DM18 and DM19 pectins have a high DB, but DM19 has a higher DB than the DM18.

3.2. Pectin supplementation influenced intestinal Th cell subsets more than systemic Th cell subsets

To study the effect of the structurally different pectins on immunological changes, mice were sacrificed after 1 week and 4 weeks of pectin administration for the collection of spleens and MLNs. These organs were used to quantify T cell subsets at systemic and intestinal level, respectively.

As shown in Fig. 1, splenic T cells and splenic cytotoxic T cell frequencies did not change by administration of any of the four pectin structures neither after 1 nor after 4 weeks of pectin administration. This

Table 1

Structural characteristics of the pectins. Pectins were characterized for the degree of methyl-esterification (DM), degree of blockiness (DB), molecular weight (Mw), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), and uronic acid (UA). (Beukema, Jermendi, van den Berg, et al., 2021).

Pectin	Origin	DB (%)	Mw	Sugar composition (mol%)					Carbohydrate content (%)
				Rha	Ara	Gal	Glc	UA	
DM18	Lemon	86	78,000	1	0	2	0	97	62
DM19	Lemon	94	75,000	1	1	3	0	95	63
DM43	Lemon	60	79,000	0	0	0	0	99	77
DM49	Lemon	33	114,000	0	1	2	0	96	73

was different for splenic Th cells which did increase after administration of the different pectins. Most pectins induced a significant increase in splenic CD4$^+$ T cells after 1 week. The DM18 (low DB) pectin was an exception as only at 4 weeks and not at 1 week increased CD4$^+$ cells frequencies were observed. The frequencies of the different CD4$^+$ subsets (Th1, Th2, Th17, and Treg) in the spleen from pectin treated mice did not differ from control mice.

In the MLNs more changes in T cell subsets were observed. Each specific pectin structure induced distinct changes in the frequencies of Th1, Th2, Th17, and Treg subsets after 1 or 4 weeks of pectin supplementation (Fig. 2) despite unchanged frequencies of Th cells and cytotoxic T cells. Th1 frequencies significantly increased after 1 week of supplementation with 51.0% ($p < 0.05$) in DM19 (high DB) pectin supplemented mice, with 37.8% ($p < 0.001$) in DM49 (low DB) pectin supplemented mice or with 60.3% ($p < 0.0001$) in DM43 (high DB) pectin supplemented mice. However, after 4 weeks of supplementation with these pectins, Th1 frequencies significantly decreased to similar levels as control mice. Supplementation with DM18 (low DB) pectin induced a different effect compared to the other pectins, as Th1 frequencies were unchanged after 1 week but increased instead of decreasing after 4 weeks of pectin supplementation. Th2 cells were also impacted by the three pectins that influenced Th1 as an increase in Th2 was observed after 1 week supplementation of DM19 (high DB) pectin (53.4%, $p < 0.05$), DM49 (low DB) pectin (49.5%, $p < 0.05$) and DM43 (high DB) pectin (65.0%, $p < 0.05$). In addition, the effects of DM18 (low DB) pectin were different here, as it did not increase Th2 frequencies. These increased Th2 frequencies went back to control numbers after 4 weeks supplementation of DM19 (high DB) and DM49 (low DB) pectin but remained significantly increased with 1.08% ($p < 0.05$) after DM43 (high DB) pectin supplementation. Moreover, Th17 and Treg frequencies were unaffected by DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins but did change after DM18 (low DB) pectin supplementation. Th17 cells were increased with 41.1% ($p < 0.05$) after 1 week of DM18 (low DB) pectin treatment, but these frequencies dropped after 4 weeks of pectin supplementation. Treg cells were enhanced with 25% ($p < 0.001$) between 1 and 4 weeks of DM18 (low DB) supplementation, but these frequencies of Tregs were not significantly different from control mice after 1 or 4 weeks of pectin supplementation. Together, the data suggest that each pectin structure distinctly and specifically impacts T cell immunity in the intestine. Pectins impact Th cell subsets more in the MLN than in the spleen.

3.3. The different pectins increase specific regulatory T cell subsets in the MLN

The effect of pectin supplementation was further studied by investigating effects on Treg subsets as these may be influenced despite the absence of effect on total frequencies of Tregs in the MLN. Tregs can be derived from the thymus (tTreg) or they can be peripherally induced (pTreg) from naïve CD4$^+$ T cells after antigen stimulation (Koizumi & Ishikawa, 2019). As shown in Fig. 3A-C, all four tested pectins did increase pTregs frequencies in the MLN after four weeks of supplementation but not of tTregs in the MLN. pTregs were enhanced for DM18 (low DB) pectin with 4.43% ($p < 0.01$), for DM19 (high DB) pectin with 4.31% ($p < 0.01$), for DM49 (low DB) pectin with 3.40% ($p < 0.01$) and

for DM43 (high DB) pectin with 4.31% ($p < 0.01$) after 4 weeks. The DM43 (high DB) pectin was the only pectin that also increased the frequencies of pTregs after 1 week of pectin supplementation (3.41%, $p < 0.05$). This was specific for the MLN as splenic pTregs and tTregs did not change after pectin supplementation.

These pTregs and tTregs can be further distinguished by the expression of specific transcription factors that are also expressed on Th1 (Tbet), Th2 (GATA3), or Th17 cells (Rorγt) (Koizumi & Ishikawa, 2019). Tbet$^+$ Tregs can be both thymus-derived and peripherally induced Tregs, GATA3$^+$ Tregs are thymus-derived Tregs, and Rorγt$^+$ Tregs are peripherally induced Tregs (Koizumi & Ishikawa, 2019). Only Rorγt$^+$ Tregs were increased after supplementation with pectins (Fig. 3). Rorγt$^+$ Tregs already increased after 1 week supplementation with 23.9% for DM49 (low DB) pectin ($p < 0.01$) and with 24.5% for DM43 (high DB) pectins ($p < 0.01$). Effects were more pronounced after 4 weeks of pectin supplementation as Rorγt$^+$ Tregs were significantly increased with 35.5% ($p < 0.01$) for DM19 (high DB) pectin, with 37.9% for DM49 (low DB) pectin ($p < 0.01$) and with 44.3% for DM43 (high DB) pectin ($p < 0.01$). DM18 (low DB) pectin showed only a trend towards increased frequencies of Rorγt$^+$ Tregs after 4 weeks of supplementation. This implies that DM19 (high DB), DM49 (low DB) and DM43 (high DB) pectin structures increase peripherally induced Rorγt$^+$ Tregs in the MLN in mice.

3.4. Pectins supplementation did not increase organic acid levels in caecum, whereas specific pectins enhanced Ahr activating components

Pectins can stimulate the production of organic acids, including SCFAs which are known to influence T cell immunity (Smith et al., 2013). Therefore, organic acid concentrations in pooled caecal digesta were measured from control or pectin supplemented mice (Fig. 4). After 1 week of pectin supplementation, total organic acids levels were lower in caecal digesta from pectin treated mice compared to control mice (control: 106 μmol mg^{-1}; DM18: 50.1 μmol mg^{-1}; DM19: 48.5 μmol mg^{-1}; DM49: 42.7 μmol mg^{-1} and DM43: 72 μmol mg^{-1}). After 4 weeks, control mice and pectin supplemented mice showed minor changes in total organic acid content compared to 1 week of pectin supplementation. Control mice showed a total organic acid content of 72.4 μmol mg^{-1}, DM18 (low DB) pectin supplemented mice 48 μmol mg^{-1}, DM19 (high DB) pectin supplemented mice 41.9 μmol mg^{-1}, DM49 (low DB) pectin supplemented mice 48.1 μmol mg^{-1} and DM43 (high DB) pectin supplemented mice 43.9 μmol mg^{-1}. There were no differences observed in organic acid composition between control and pectin treated mice after 1 or 4 weeks of pectin supplementation. These results indicate that the pectin supplementation does not enhance the production of succinic acid, lactic acid or the SCFAs acetate, butyrate, or propionate in the caecum.

In addition to SCFAs, other microbial-derived metabolites such as tryptophan metabolites (indole derivatives) can enhance T cell immunity by activating the aryl hydrocarbon receptor (Ahr) (Lamas, Natividad, & Sokol, 2018; Ye et al., 2017). Therefore, the Ahr activating properties of caecal samples from mice supplemented with different pectins were measured using a reporter cell line that expresses Ahr. As shown in Fig. 5, Ahr was only activated by caecal digesta from mice supplemented with specific pectin structures. After 1 week of pectin
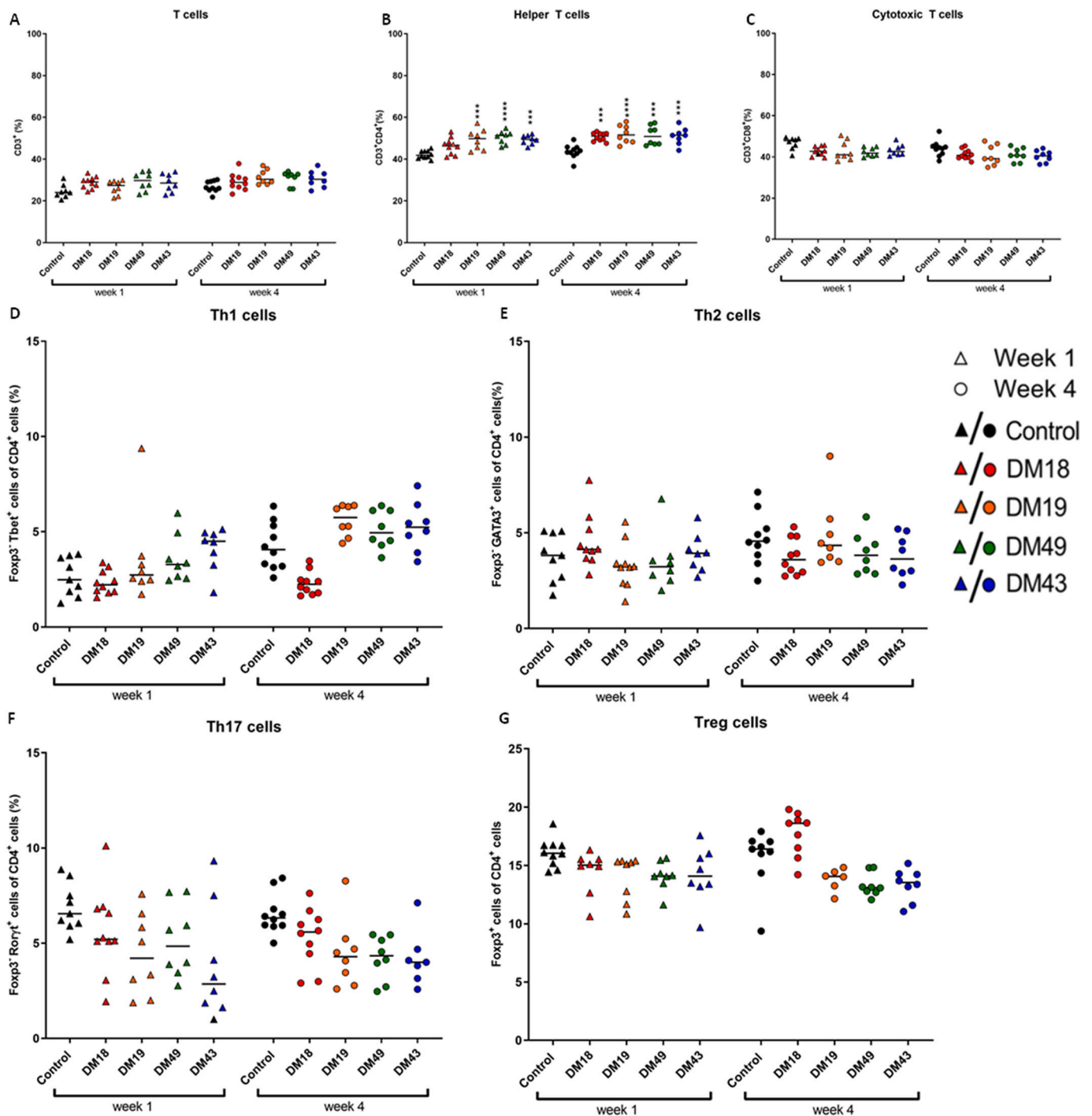
Fig. 1. T cell frequencies of the spleen after 1 and 4 weeks of pectin supplementation. Frequencies of T cells (A), helper T cells (B), cytotoxic T cells (C), Th1 cells (D), Th2 cells (E), Th17 (F), and regulatory T cells (G) in the spleen of control or pectin supplemented mice. Significant differences 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 **** $p < 0.0001$). ($n = 8$ per experimental group).

supplementation, a significant increase in Ahr activation was measured after stimulation with digesta from DM19 (high DB) pectin (47.36% vs. control, $p < 0.0001$) and DM49 (low DB) pectin (36.72% vs. control, $p < 0.05$) supplemented mice. In addition, Ahr was also activated after stimulation with caecal digesta samples from mice that were supplemented with DM19 (high DB), DM49 (low DB) and DM43 (high DB) pectins for 4 weeks (DM19: 56.86% vs. control, ($p < 0.0001$); DM49: 49.72% vs. control, ($p < 0.01$); DM43: 45.0% vs. control, ($p < 0.05$)). Together these results suggest that pectin supplementation in the

current study does not enhance SCFA production, but that DM19 (high DB), DM49 (low DB) and DM43 (high DB) pectin structures enhance Ahr-activation in the caecum of mice.

3.5. The impact of pectin supplementation on the caecal microbiota composition

To study the impact of the different pectin structures on the intestinal microbiota composition, 16S rRNA gene amplicon sequencing was
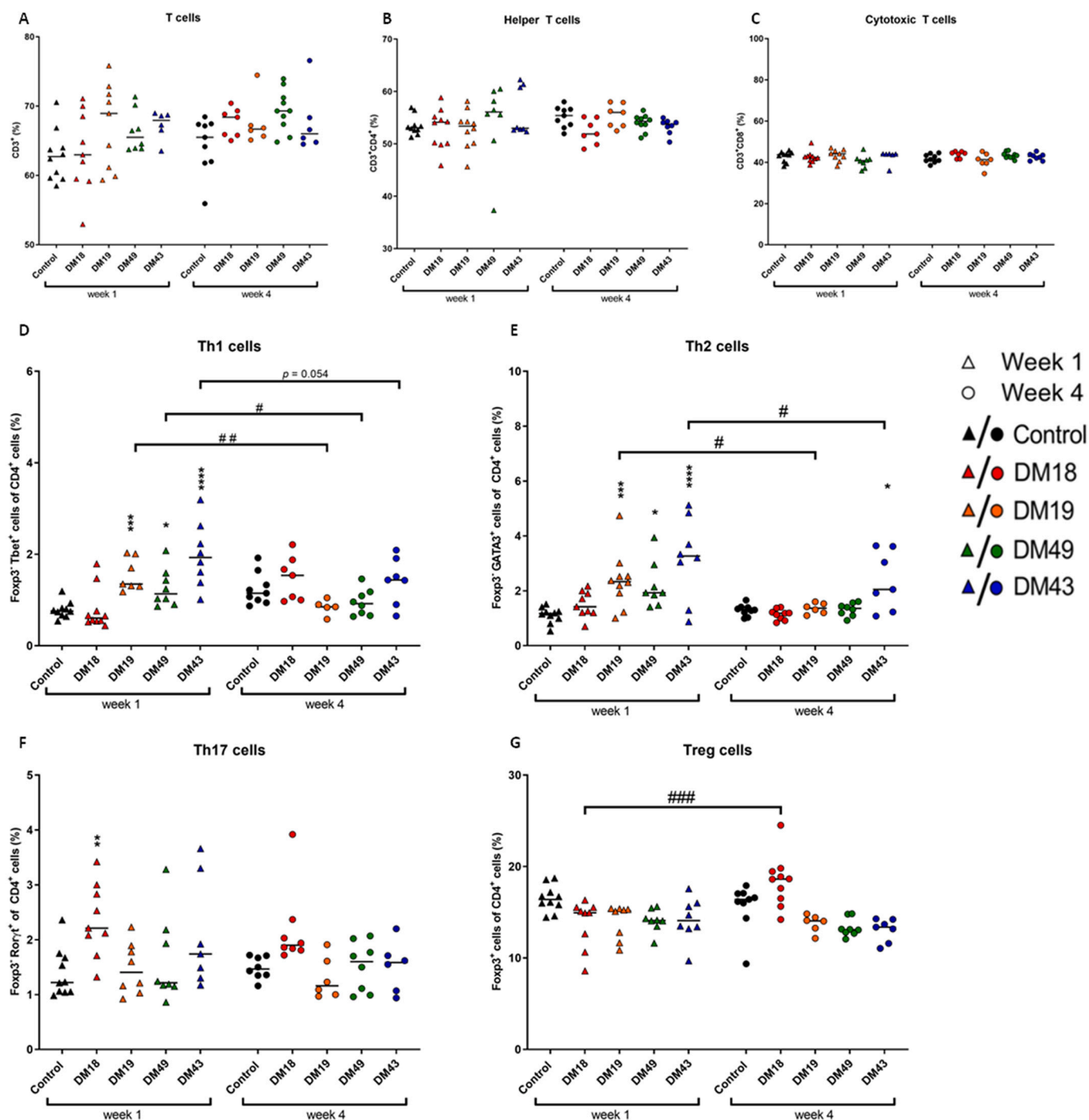
Fig. 2. T cell frequencies of the MLN after 1 and 4 weeks of pectin supplementation. Frequencies of T cells (A), helper T cells (B), cytotoxic T cells (C), Th1 cells (D), Th2 cells (E), Th17 cells (F), and regulatory T cells (G) in the MLN of control or pectin supplemented mice. Statistically significant 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 # (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$).

performed. Some genera were influenced by all pectins, whereas other genera were changed by specific pectins (Fig. 6, Supplementary Table 3). The DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins supplementation lead to a relatively similar microbiota composition in mice after 1 and 4 weeks of pectin supplementation, but this was different from the microbiota composition of control mice or mice supplemented with the DM18 (low DB) pectin (Fig. 6, Supplementary Table 3). After 1 week of pectin supplementation, the relative abundance of *Muribaculaceae* spp. was 3.66% lower for DM19 (high DB), 7.93% for DM49 (low DB), and 13.58% for DM43 (high DB) pectins

compared to control mice, whereas the relative abundance of this genus was 1.62% higher by DM18 (low DB) supplementation. The 4 weeks of pectin supplementation led to even more differences in microbiota composition between DM19 (high DB), DM49 (low DB), DM43 (high DB) pectins and the DM18 (low DB) pectin. Compared to control mice, the relative abundance of the genera *Lachnospiraceae NK4A136*, *Desulfovibrionaceae* spp., and *Alloprevotella* were strongly increased by DM19 (high DB) pectin with a respective 12.25%, 3.58%, and 2.57%, DM49 (low DB) pectin with a respective 9.34%, 1.44%, and 1.23%, and DM43 (high DB) pectin with a respective 11.58%, 1.17%, and 0.85%. However,
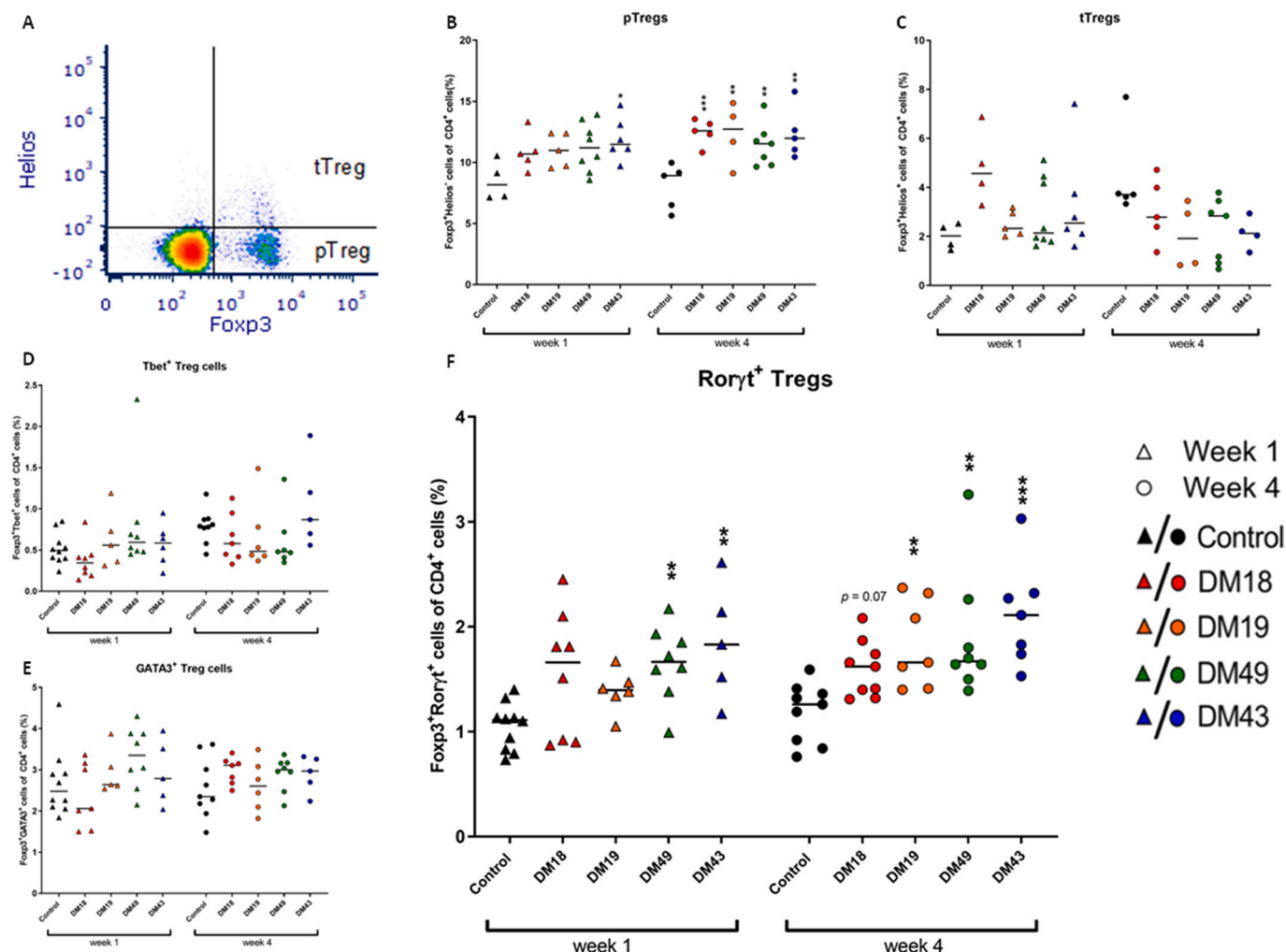
Fig. 3. Regulatory T cell frequencies of the MLN after 1 and 4 weeks of pectin supplementation. Peripheral induced Tregulatory cells (pTreg) and Thymus derived Tregulatory cells (tTreg) were selected by plotting Foxp3 and Helios from CD3$^+$CD4$^+$ T cells (A). Frequencies of pTregs (B), tTregs (C), 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 #.
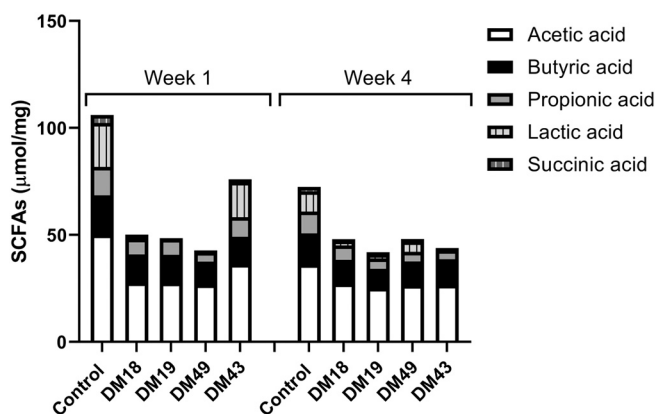

Fig. 4. Amount of organic acid profiles in caecal digesta. The amount of SCFAs in pooled caecal digesta samples from control mice or from mice supplemented with DM18, DM19, DM49 or DM43 pectins.

the relative abundance after DM18 (low DB) pectin supplementation were only 2.52% higher for *Lachnospiraceae NK4A136,* and respectively 1.32% and 0.65% lower for *Desulfovibrionaceae* spp. and *Alloprevotella*.

On the contrary, the relative abundance of the genera of *Lactobacillus* and *Lachnospiraceae* spp. were respectively 4.21% and 5.2% higher in DM18 (low DB) pectin supplemented mice, whereas the relative abundance was decreased by supplementation of the other pectin structures. These studies demonstrate, therefore, that the DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins change the microbiota composition in mice differently than DM18 (low DB) pectin.

To investigate which specific genera may positively correlate to the pectin-induced increase of Th1 levels, Th2 levels, and Rorγt$^+$ Treg frequencies, we performed a Spearman correlation test (Table 2). As the genus *Lachnoclostridium* significantly correlated ($p < 0.05$, FDR < 0.05) with Th1 and Th2 levels, the increase in the abundance of *Lachnoclostridium* may be responsible for the increase in Th1 and Th2 levels after 1 week of DM19 (high DB), DM49 (low DB), DM43 (high DB) pectin supplementation. Furthermore, the relative abundance of *Lachnospiraceae NK4A136* ($p < 0.01$, FDR < 0.05) and *Ruminococcaceae UCG-003* ($p < 0.05$, FDR < 0.05) positively correlated to Rorγt$^+$ Treg levels. The relative abundance of *Lachnospiraceae NK4A136* did also change drastically by DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectin supplementation (Fig. 6), suggesting that the increase in relative abundance of *Lachnospiraceae NK4A136* may be responsible for the increase in Rorγt$^+$ Treg frequencies. This was not the case for *Ruminococcaceae UCG-003*.
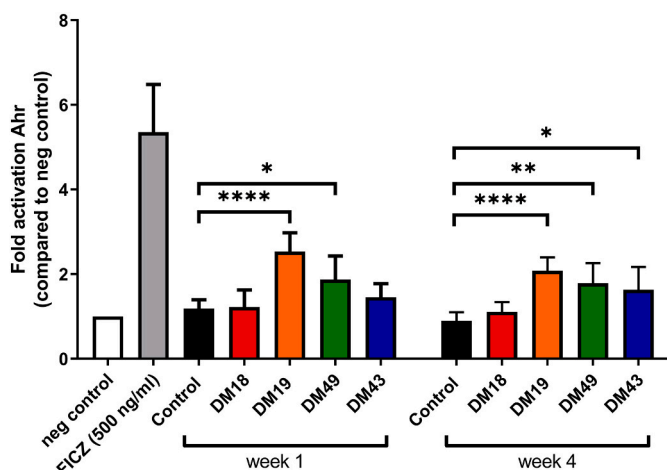
Fig. 5. Presence of Ahr ligands in caecal digesta. The presence of indole derivatives in caecal digesta samples was measured with an aryl hydrocarbon-reporter (Ahr) reporter cell line. Caecal digesta from control mice or from mice supplemented with DM18 (low DB), DM19 (high DB), DM49 (low DB) or DM43 (high DB) pectins was tested for Ahr activating properties. Statistical significant differences are indicated by * (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$).

4. Discussion

Several studies have demonstrated that pectin beneficially influences intestinal immunity and prevents intestinal inflammation and diseases (Ishisono, Mano, Yabe, & Kitaguchi, 2019; Jiang et al., 2016; Sahasrabudhe et al., 2018; Sun et al., 2017; Wu et al., 2019). The exact mechanisms responsible for these protective effects of pectins are not fully understood and it is also unknown which structural features of pectins are responsible for beneficial effects. The current study demonstrates that beneficial effects are not derived from a general characteristic of pectins but that pectins with a specific DM and DB are responsible for distinctive modulation of T cell intestinal immunity in mice.

The current study shows that supplementation with DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins influences Th1, Th2 and Rorγt⁺ Treg cell immunity in mice, whereas this was not influenced by DM18 (low DB) pectins which are known to have inhibiting effects on innate immune receptors such as TLR2–1 (Beukema, Jermendi, Koster, et al., 2021; Sahasrabudhe et al., 2018) and induce anti-inflammatory effects in mice with TLR2-mediated mucositis (Beukema, Jermendi, van den Berg, et al., 2021). Both DM18 (low DB) pectin and DM19 (high DB) pectins showed a similar level of TLR2–1 inhibition, which can be related to the relatively similar DM and DB of these pectins (Beukema, Jermendi, Koster, et al., 2021). In the current study these pectins showed, however, a different impact on T cell immunity, which can only be explained by the small difference (8%) in DB. The DM19 (high DB) pectin, that had an impact on T cell immunity, has a little more blockwise distribution of non-esterified GalA residues than the DM18 (low DB) pectin, which had no impact on T cell immunity. This difference in the number of blockwise distributed non-esterified GalA residues may however be negligible as both DM18 (DB84%) and DM19 (DB94%) possess very high level of blockwise distributed non-esterified GalA residues (Beukema, Jermendi, Koster, et al., 2021). It is more likely that methyl-esterified GalA residues play a role in the impact on T cell, since pectins with more methyl-esterified GalA residues (DM49 and DM43) also show effects on T cell immunity.

Our data demonstrate that the low dose of DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins specifically induced a different microbiota composition compared to the DM18 (low DB) pectin after 1 and/or 4 weeks of pectin supplementation. A difference was observed for *Lachnospiraceae NK4A136, Desulfovibrionaceae unidentfied, Alloprevotella, Lactobacillus,* and *Lachnospiraceae* spp.. Microbes from Lachnospiraceae, Desulfovibrionaceae, *Lactobacillus* and *Alloprevotella* are known to be enhanced after pectin stimulation (Tian, Scholte, et al., 2016; Zhu et al., 2020), and may utilize pectins using enzymes, such as pectin lyase, pectin methylesterase, and polygalacturonase that facilitate the breakdown of pectin molecules (Dongowski et al., 2002). A difference in structural patterns between the pectins may however be responsible for the different microbial stimulating effects. Such structural dependent effect of pectins on microbiota composition was also
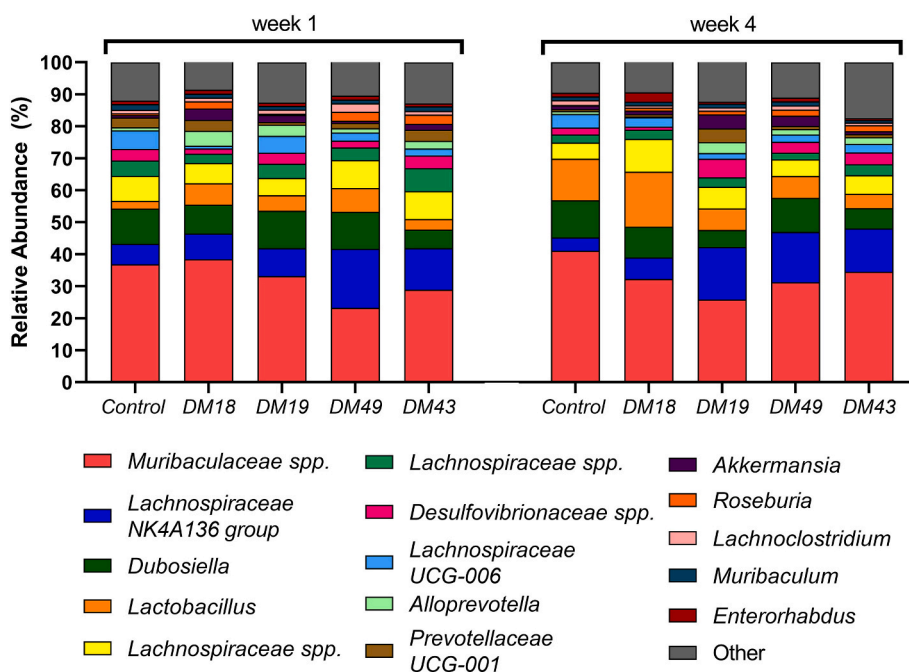

Fig. 6. Relative abundance genera in the microbiota of mice in the control or pectin groups. The relative abundance of different microbes on genus level was determined from pooled caecal digesta of control mice or from mice supplemented with DM18 (low DB), DM19 (high DB), DM49 (low DB) or DM43 (high DB) pectins for 1 or 4 weeks.

Table 2
Relative abundance of genera that positively correlate significantly with Ahr activation or Rorγt⁺ Treg levels.

Taxon family	Taxon genus	Relative abundance (%)										Correlation with Th1 levels	Correlation with Th2 levels	Correlation with Rorγt⁺ Treg levels
		Week 1					Week 4							
		Control	DM18	DM19	DM49	DM43	Control	DM18	DM19	DM49	DM43			
Lachnospiraceae	*Lachnoclostridium*	1.05	1.11	1.26	1.12	2.65	1.44	0.70	0.98	0.82	1.34	*	*	ns
Lachnospiraceae	*Lachnospiraceae NK4A136*	6.45	8.03	8.75	13.02	18.43	4.16	6.68	16.41	13.50	15.74	ns	ns	**
Ruminococcaceae	*Ruminococcaceae UCG-003*	0.00	0.17	0.21	0.19	0.21	0.00	0.00	0.22	0.22	0.40	ns	ns	**

Significant correlation was determined with Spearman's rank correlation test. Correlation was considered significant when the absolute value of Spearman's rank correlation coefficient (Spearman's r) was >0.6 and statistically different when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$ and $p < 0.001$). Significance was defined by FDR < 0.05. ns = not significantly different.

found for the DM difference in low and high DM pectins, where the low number of methyl-esterified GalA residues in low DM pectins induced different alterations of specific microbes than pectins with higher numbers of methyl-esterified GalA residues (Dongowski et al., 2002; Tian, Scholte, et al., 2016). In the tested pectins, a difference in distribution of methyl-esterified GalA residues might be responsible for the different effects in low DM pectins. The low DM pectins with a high DB might have a higher level of blockwise distributed methyl-esterified GalA residues compared to low DB pectins as the blocks of non-esterified GalA residues may also cluster the methyl-esterified GalA residues (Jermendi et al., 2022). These blocks of methyl-esterified GalA residues in the high DB pectin may be differently fermented by microbial derived enzymes compared to the low DB pectin. However, a more in-depth analysis to the distribution of methyl-esterified GalA residues and non-esterified GalA residues (Jermendi et al., 2022) might give more insight in whether the presence of blockwise distributed methyl-esterified GalA residues may play a role in the effects on microbiota composition.

It is however unlikely that the pectins are used to significantly enhance the growth of the microbes as the dose (3 mg/day) of pectins constitute 0.1% of the daily food intake of mice (Bachmanov, Reed, Beauchamp, & Tordoff, 2002), which is much lower than 3% in the study of Tian et al. (Dongowski et al., 2002; Tian, Scholte, et al., 2016). It is more likely that the low dose of the specific pectin structures initially stimulate the growth of these microbes, which further used other nutritional components in the food, such as arabinoxylan or β-glucan (Tian et al., 2019), as growth substrate. This can also explain the lack of SCFA enhancement after pectin supplementation, which was found after supplementation with a higher dose of pectin (Dongowski et al., 2002; Tian, Scholte, et al., 2016). Together, these studies suggest that the supplementation of DM19 (high DB), DM49 (low DB), and DM43 (high DB) initiates a different microbiota composition compared to the DM18 (low DB) pectin, which might result in a different stimulation of specific microbial communities that may have the ability to induce T cell immunity.

Supplementation of DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins for 1 week induced an increase in Th1 and Th2 frequencies, which returned to control levels after 4 weeks supplementation of these pectins. These increased frequencies of Th1 and Th2 significantly correlated to an increased level of *Lachnoclostridium*, which suggests that microbes from *Lachnoclostridium* have immune-stimulatory effects and may be responsible for the Th1 and Th2 inducing effects. The exact mechanism through which *Lachnoclostridium* exerts Th1 and Th2-stimulating effects need to be further explored, but it may derive from *Lachnoclostridium*-derived antigens or metabolic products, as these compounds were found to enhance Th1 and Th2 responses (Berer et al., 2018; Eberl, 2016).

Our data demonstrate that supplementation with the DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins also increased the generation of Rorγt⁺ Tregs in the MLN. The stimulation of the generation of Rorγt⁺ Tregs is dependent on the intestinal microbiota composition (Yang et al., 2016). Specific microbiota species can enhance the generation of Rorγt⁺ Tregs through enhancing the production of microbial-derived metabolites (Hussein et al., 2020; Lozano-Ojalvo et al., 2019; Ohnmacht et al., 2015; Song et al., 2020). The microbial-derived metabolites that enhance Rorγt⁺ Tregs are probably not SCFAs as there was no enhancement of SCFA profiles measured after pectin treatments in the administered pectin concentrations. This is in line with previous findings showing no enhancement of SCFAs after pectin supplementation in this pectin dose in mice (Sahasrabudhe et al., 2018). It is more likely that microbial-derived metabolites which activate the aryl-hydrogen receptor (Ahr) are responsible for the generation of Rorγt⁺ Tregs in these mice, because our data showed high activation of Ahr after stimulation with digesta from DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectin supplemented mice. The relative abundance of genera *Lachnospiraceae NK4A136* increased drastically

and correlated positively to Rorγt$^+$ Treg levels, suggesting that microbes from these genera may be responsible for the production of molecules with Ahr activating properties. Microbes from Lachnospiraceae were correlated before to the production of indole derivatives, which are tryptophan converted metabolites with Ahr activating properties (Amaretti et al., 2019; Vacca et al., 2020). These tryptophan metabolites may stimulate the Ahr on Rorγt$^+$ Tregs, which are highly expressed by these cells in the intestine (Ye et al., 2017), and stimulate the expansion of Rorγt$^+$ Tregs as was observed in our study (Ye et al., 2017). Yet, we did not find a strong correlation between the increase in Ahr activating components in the cecal digesta and the increase in this Lachnospiraceae NK4A136. However, the relative abundance does not reveal the absolute load of Lachnospiraceae NK4A136 in caecal digesta which might correlate to a significant increase in Ahr activation. Future studies should therefore focus on the absolute abundance of microbiota composition besides the relative abundance. Collectively, our findings suggest that the 4 weeks of supplementation of DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins increases the relative abundance of *Lachnospiraceae NK4A136* which may produce large amounts of indole derivatives which leads to the generation of Rorγt$^+$ Tregs cells (Amaretti et al., 2019; Vacca et al., 2020).

The current study found that an increase in Rorγt$^+$ Tregs coincides with a decrease in Th1 and Th2 responses after 4 weeks of pectin supplementation. Rorγt$^+$ Tregs play an important role in suppressing immune responses of effector T cells and are reported to prevent e.g. the development of colitis (Britton et al., 2019; Yang et al., 2016). Ohnmacht et al., 2015 demonstrated that Rorγt$^+$ Tregs have suppressive functions on Th2 cell responses (Ohnmacht et al., 2015). Another study also showed that lack of Rorγt$^+$ Tregs increases Th1 and Th17 responses (Sefik et al., 2015). These studies suggest that the increasing frequencies of Rorγt$^+$ Tregs after supplementation of DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins may be responsible for the suppression of Th1 and Th2 responses after 4 weeks of pectin supplementation, whereas the 1 week supplementation of the pectins may not sufficiently increase Rorγt$^+$ Tregs to reduce the pectin-induced increase in Th1 and Th2 cells. Together, our results suggest that supplementation with DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectins enhances the generation of Rorγt$^+$ Tregs which may suppress Th1 and Th2 responses after 4 weeks of pectin supplementation.

5. Concluding remarks

In the current study, we hypothesized that pectin influences T cell immunity in a DM and DB dependent manner through modulation of the intestinal microbiota. Our study demonstrates that supplementation with a low dose of DM19 (high DB), DM49 (low DB) or DM43 (high DB) pectins induces the generation of Th1, Th2, and Rorγt+ Tregs in mice. This increase in Th1 and Th2 frequencies may be induced by an increased relative abundance of Lachnoclostridium after 1 week of pectin supplementation. The increased frequencies of Rorγt+ Tregs may relate to an enhanced production of Ahr activating metabolites from Lachnospiraceae NK4A136 and Ruminococcaceae UCG-003 after 4 weeks of pectin supplementation. Besides, these Rorγt+ Tregs may reduce Th1 and Th2 levels back to control levels after 4 weeks of pectin supplementation. This knowledge may be important for the design of functional foods with anti-inflammatory properties. Consumers may benefit from consuming a low dose of DM19 (high DB), DM49 (low DB) and DM43 (high DB) as the increased levels of Rorγt+ Tregs may prevent the development of Th1 and Th2-mediated intestinal diseases, such as Crohn's disease and Ulcerative colitis (Britton et al., 2019; Imam et al., 2018)), and restore intestinal homeostasis (Eberl, 2016).

CRediT authorship contribution statement

M. Beukema: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft. **É.**

Jermendi: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **M.M.P. Oerlemans:** Data curation, Formal analysis, Investigation, Writing – review & editing. **M.J. Logtenberg:** Data curation, Formal analysis, Investigation, Writing – review & editing. **R. Akkerman:** Data curation, Formal analysis, Investigation, Writing – review & editing. **R. An:** Data curation, Formal analysis, Investigation, Writing – review & editing. **M.A. van den Berg:** Resources, Conceptualization, Investigation, Writing – review & editing. **E. G. Zoetendal:** Formal analysis, Investigation, Writing – review & editing. **T. Koster:** Data curation, Formal analysis, Investigation. **C. Kong:** Data curation, Formal analysis, Investigation. **M.M. Faas:** Supervision, Validation, Writing – review & editing. **H.A. Schols:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing. **P. de Vos:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119280.

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