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Structure-function relationship between homogalacturonan pectins and intestinal immunity

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CHAPTER 8

Discussion

Lower intake of dietary fibers has been correlated to a higher incidence of immune-related diseases in Western countries [1,2]. These diseases occur at low frequencies in societies that consume high-fiber diets, suggesting that dietary fibers ingestion beneficially influences the immune system of the host [1,2]. Considering that the gut-associated lymphoid tissue (GALT) comprises a major part of the immune system [3], it is very likely that dietary fibers prevent the development such immune related diseases by acting on the immune system in the gastrointestinal tract. The impact of fiber ingestion on health is however strongly determined by the composition of the different types of dietary fibers [4].

One type of dietary fibers that are recognized for their impact on health are pectins, which are cell wand derived dietary fibers isolated from several fruits and vegetables [5,6]. Epidemiologic evidence has demonstrated that the consumption of pectins alleviates colonic inflammation and limits the development of type 2 diabetes, obesity and cancer [7,8]. Many mechanisms through which pectins act on health have been proposed, including reducing the absorption of glucose and LDL cholesterol, increasing satiation, or trapping of bile acids and other carcinogenic substances [8]. Besides these mechanisms, pectins are also recognized as immunomodulating agent and are considered to influence intestinal immunity through direct interactions or through microbiota-dependent effects [9]. How pectins impact intestinal immunity is dependent on structural characteristics of pectins [9]. Commercial pectin molecules consist largely (>60%) of homogalacturonan regions, but they can also contain rhamnogalacturonan I, rhamnogalacturonan II, xylogalacturonan or apiogalacturonan regions [10]. Homogalacturonan regions can be methyl-esterified on the galacturonic acid backbone. Most studies investigating the impact of pectins on intestinal immunity used pectins that consisted of a combination of these structural regions (**Chapter 1)**. Therefore, little is known about the impact of individual or specific structural features of pectins on intestinal immunity. Homogalacturonan regions in pectins may have the strong potential to play a role in the immunomodulatory effects of pectins as pectin are composed largely of these structural regions (~60%) [10]. Additionally, the level and distribution of methyl-esters in homogalacturonan pectins strongly impact the functional properties of pectins in food processing [11]. However, whether the degree and distribution of methyl-esters of homogalacturonan pectins influence intestinal immunity was until start of the studies presented in this thesis unknown. Elucidating

which specific structural characteristics of pectins impact intestinal immunity may be of importance, as it will provide knowledge that is essential for the design of a better and more tailored nutritional formulation with health promoting effects.

The aim of this thesis was to unravel the immune modulating effects of homogalacturonan pectins that varied in the degree and distribution of methyl-esters on the gastrointestinal immune system. Both the direct and the microbiota-dependent impact of homogalacturonan pectins on intestinal immunity were investigated. Direct effects of pectins were determined by screening the impact of structurally different pectins on Toll-like receptor (TLR) signaling and TLR-mediated inflammation of the small intestine in mice with doxorubicin-induced mucositis. First, the impact of the degree of methyl-esterification (DM) of homogalacturonan pectins on TLR signaling was determined. Then, the combined impact of the degree and distribution of methyl-esters (degree of blockiness; DB) of homogalacturonan pectins on TLR signaling and TLR-mediated inflammation was studied. After this, microbiota-dependent effects were studied by testing homogalacturonan pectins that differed in degree (DM) and distribution of methyl-esters (DB) in healthy mice. The impact of the pectins on the interaction between microbiota and immunity was determined by studying the microbiota composition, fermentation profiles, and on T cell frequencies. Then, we investigated *in vitro* and *in vivo* how the impact of pectins on the intestinal microbiota may prevent colonic inflammation induced by the enteric pathogen *Citrobacter rodentium*.

Regional composition of the immune system and the presence of microbiota determine the bioavailability of pectins along the gastrointestinal tract

Along the gastrointestinal tract there are compositional differences of the immune system and differences in the abundance of microbiota species [12,13] which both can impact the availability of pectins to impact the intestinal immune system. The small intestine consists of a thin and loose mucus layer, but the thickness of the mucus layer increases towards distal sides of the gastrointestinal tract [14]. The mucus layer in the large intestine consists of an additional dense and thick layer [14]. The loose mucus layer in the small intestine allows direct interaction of luminal substances, such as pectins, with immune cells and immune receptors [15], whereas a thick layer in the large

intestine prevents this direct interactions of large luminal substances with the immune system [13]. Therefore, pectins may directly interact with the intestinal immune system in the small intestine, but not in the large intestine as the thick mucus layer prevents this interaction.

Besides the regional differences in the composition of the immune system, there is also a large difference in the abundance of different species of intestinal microbiota along the gastrointestinal tract [12]. The intestinal microbiota is present in low abundance at proximal sides of the small intestine, but the abundance of intestinal microbiota species increases towards distal sides of the gastrointestinal tract [16]. This may results in a different regional utilization of pectins along the gastro-intestinal tract as was confirmed in several studies. Saiko et al., 2005 [17] and van Trijp et al., 2020 [18] demonstrated that the microbiota from the small intestine is not very efficient in degrading pectins. This resulted in a recovery of 90% of ingested pectins from the terminal ileum in healthy human subjects [17]. In contrast, the microbiota in the large intestine is very efficient in utilizing pectins [19] which resulted in large production of fermentation products, such as anti-inflammatory SCFAs [19,20]. The digestion of pectins by microbial species may be undesirable for the direct effects of pectins on intestinal immunity, because pectins digested by microbial enzymes lack the ability to induce direct effects on immune receptors, whereas undigested intact pectins can induce immunomodulatory effects on immune receptors [21]. Collectively, these studies suggest that intact pectin molecules induce direct effects in the small intestine, considering that these intestinal segments contain thin mucus layers and a low abundance of pectin-degrading microbes. In the large intestine, a thick mucus layer and high abundance of pectin-degrading microbes prevent direct interactions of pectins with immune cells. Here, the effects of pectins on intestinal immunity may derive from microbiota-dependent effects.

Homogalacturonan pectin structures induce direct effects on TLR signaling

Dietary fibers are known to influence innate immune responses through direct interactions with pattern recognition receptors [22]. Toll-like receptors (TLRs) are the best characterized pattern recognition receptors expressed in the intestine that have been identified to recognize dietary fibers and influence intestinal immune cells [23–25].

Several studies have shown that pectins influence TLR signaling [26,27], but how the DM and DB of homogalacturonan pectins influence TLR signaling has not been investigated yet. Therefore, we screened homogalacturonan pectins that differed in DM (**Chapter 2**) or DM and DB (**Chapter 3**) on TLR activating and TLR inhibiting properties. The tested homogalacturonan pectins induced no or minimal activation of the different TLRs, but were rather efficient in inhibiting TLR activation. Of the different TLRs, TLR2-1 was mainly inhibited by the homogalacturonan pectins and minimal inhibition of other TLRs was observed by these pectins. This was in contrast to other studies which observed that TLR2-6, TLR4, and TLR9 were efficiently inhibited by pectins rich in RG-I regions [26,28]. These studies confirm that the potency of pectins to activate or inhibit different TLRs depends on structural characteristics of pectins. Homogalacturonan pectins induce more inhibitory effects on TLR2-1, whereas pectins containing RG-I may have the potency to inhibit TLR2-6, TLR4, and TLR9.

The impact of homogalacturonan pectins on TLR2-1 inhibition is strongly influenced by the level and degree of methyl-esterification. In **Chapter 2**, we showed that pectins with a lower DM increased the TLR2-1 inhibiting properties of pectins compared to pectins with a higher DM. These findings show that a larger number of non-esterified GalA residues in low DM pectins induce strong TLR2-1 inhibiting effects. Moreover, in **Chapter 3**, we confirmed this high inhibition of TLR2-1 by low DM pectins, but we also found that intermediate DM (~DM46) pectins with a high DB inhibit TLR2-1 as strong as low DM pectins (~DM19). However, intermediate DM pectins with a low DB or very high DM pectins (~DM86) did not inhibit TLR2-1 strongly. The very high DM pectin with a high DB did also not enhance the TLR2-1 inhibiting effects of the pectin. Together, these findings show that pectins with a high number and blockwise distribution of non-esterified GalA residues strongly inhibit TLR2-1 activation.

The specificity of these pectin structures to prevent TLR2-1 activation may be derived from their affinity for the TLR2 receptor. **Chapter 2** and **3** demonstrated that the low DM pectins and the intermediate DM pectins with a high DB bound stronger to the TLR2 protein compared to intermediate DM pectins with a low DB or to high DM pectins. Pectins with a blockwise distribution (high DB) and a large number of non-esterified GalA residues (low DM) have larger areas with negative charge (higher charge density) compared to pectins with a random distribution (low DB) or low

number (high DM) of non-esterified GalA residues [29]. Therefore, we hypothesized that the interaction between pectins and TLR2 is established through electrostatic interactions. As these pectin structures specifically prevented TLR2-1 dimerization and not TLR2-6, we also hypothesized that pectins bound strongly to the ligand binding site and at the interface for TLR2-TLR1 dimerization [30]. We confirmed these hypothesises in **Chapter 2** by developing mutant TLR2 ectodomains in which four positively charged amino acids R315, R316, R321 and K347 of the TLR2 ectodomain were replaced with an uncharged glutamine (Q). We developed a TLR2 (RRQQ) ectodomain which changed the positively charged amino acids of the TLR2-TLR1 binding interface (R321 and K347) to a neutrally charged amino acids [30]. We also developed the TLR2 (QQQQ) ectodomain which changed the positively charged amino acids of TLR2-TLR1 binding interface (R321 and K347) and the ligand binding site (R315 and R316) to a neutrally charged amino acid [30]. When we tested these ectodomains on a low DM pectin (DM7), we found that the pectin could bind weaker to the QQQQ than to the RRQQ mutant, showing that a more positively charged TLR2 ectodomain binds stronger to negatively charged pectins. Together, these findings suggest that the negative charged areas in low DM pectins and the intermediate DM pectins with a high DB bind to positively charged amino acids in the TLR2 ectodomain. Through this interaction, pectins may prevent the binding of the ligand to TLR2 and the dimerization of TLR2 with TLR1. Future studies should investigate which size of these negative charge areas of non-esterified GalA residues is required for TLR2 binding, which may lead to more tailored pectin molecules with strong abilities to inhibit TLR2-1.

Homogalacturonan pectin structures limit small-intestinal inflammation through direct inhibition of TLR2-1

As pectins remain largely intact in the small intestine due to the low abundance of microbial species and the thin loose mucus layer (**Chapter 1**), we hypothesized that the TLR2-1 inhibiting effects of pectins occur mostly in the small intestine. To confirm this hypothesis, we tested in **Chapter 2** and **4** the anti-inflammatory impact of TLR2-1 inhibiting pectin structures in mice with doxorubicin induced mucositis, which is a mouse model with TLR2-activated inflammation of the small intestine [31]. In **Chapter 2**, the anti-inflammatory properties of one pectin (DM7) with strong TLR2-1

inhibiting properties was tested and confirmed to exert anti-inflammatory effects at a similar level as the TLR2 blocking antibody. These findings demonstrated that a pectin with strong TLR2-1 inhibiting properties prevented the development of doxorubicin-induced mucositis. However, whether this effect was specific for TLR2-1 inhibiting properties of pectins or a general effect of the pectins was unknown from this study. Therefore, we administered four different pectin structures to mice with doxorubicin-induced mucositis in **Chapter 4**. Of these pectins, three pectins had strong TLR2-1 inhibiting properties (two low DM pectins (DM18 and DM19), with a respective low and high DB, and an intermediate DM pectin (DM43) with a high DB). We also tested one pectin with weak TLR2-1 inhibiting properties (intermediate DM (DM49) with a low DB) (**Chapter 3** and **4)**. Our data showed that the strong TLR2-1 inhibiting pectins were most efficient in preventing the development of mucositis, whereas the weak TLR2-1 inhibiting pectin showed lower anti-inflammatory effects. These findings show that the direct effects of pectin structures on TLR2-1 inhibition, as measured *in vitro*, can be extrapolated to anti-inflammatory properties of these pectin structures on small-intestinal inflammation *in vivo*.

The pectins may exert the anti-inflammatory effect on TLR2 during specific phases of the inflammatory process of mucositis. Chemo chemically-induced mucositis is characterized by several phases, including the initiation phase, the signaling and amplification phase, the ulceration phase, and healing phase [32]. During the initiation phase, the chemotherapeutic agents induce initial damage to the epithelium in the intestinal lining, which is characterized by DNA and mitochondrial damage. In response, the mitochondria produces high levels of reactive oxygen species which induce the release of intracellular damage associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) [33], or heat shock protein 60 and 70 [34,35]. The release of such molecules lead, in the signaling and amplification phase, to high activation of Nf-κB pathways [32], including the activation of TLR2 [33–35]. The high TLR2 activation lead to more apoptosis of the intestinal epithelium [31]. Apoptotic epithelial cells decrease the integrity of the epithelial lining, leading to the translocation of luminal bacteria [36]. This leads to more inflammation and the development of ulcers in the ulceration phase. After the ulceration phase, the tissues restore to their initial state in the healing phase which starts around 2 weeks after the chemotherapeutic treatment [32]. The data from **Chapter 4** suggests that pectins act on preventing the initiation of this TLR2-induced apoptosis of epithelial cells in the

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signaling and amplification phase. The anti-apoptotic properties and the barrier protective properties of pectins on the intestinal epithelium, as was found in **Chapter 6**, suggest that the pectins act on epithelial cells [21]. Preservation of epithelial integrity might prevent the translocation of intestinal bacteria to underlying tissues and subsequent inflammation and tissue damage [36,37]. In addition to epithelial cells, the pectins may also inhibit TLR2 activation in dendritic cells or macrophages (**Chapter 2)**. These immune cells play an important role in inflammation in mucositis [38], and inhibition of these responses may contribute to the anti-inflammatory effect. However, it remains elusive whether the tested large pectin molecules can influence these immune cells through direct interactions in the lamina propria or whether they influence immunity by remaining in the lumen. Future studies should therefore investigate whether the large pectin molecules are directly taken up by M cells in Peyer's patches and underlying tissues as was found for pectin oligosaccharides [39], or whether pectins remain in the lumen and influence TLR2 signaling on epithelial cells and induce anti-inflammatory effects on dendritic cells through interaction with their dendrites [40].

The anti-inflammatory effects of the pectins on doxorubicin-induced mucositis were independent of SCFA concentrations in these mice as pectin treatment did not enhance SCFA levels in the caecum of doxorubicin treated (**Chapter 2**) or in healthy mice (**Chapter 5**). SCFA levels can, however, play a role in anti-inflammatory effects in mucositis as a 15% pectin diet reduced intestinal inflammation in mice with the chemotherapeutic agent irinotecan [41]. This was mainly through the acetate production in the colon, which was suggested to prevent inflammation through binding of G-protein receptors (GPRs) or act on histone-deacetylases in immune cells [41]. In **Chapter 2** and **4**, we used however a dose of 3 mg/day, which constitute approximately 0.1% of the daily food intake [42]. The low dose of pectins may be insufficient to increase SCFA levels in the gastrointestinal tract, but may be sufficient to induce direct effects on small intestinal immunity through interaction with TLR2. Patients undergoing chemotherapeutic treatment may, however, also benefit from the consumption of a higher dose of these pectins as the anti-inflammatory effects of the enhanced production of SCFAs in the colon may add up to the direct effects of the pectins on immunity [41]. Future studies should therefore investigate whether a high dose of the structural different pectins can enhance anti-inflammatory effects on mucositis through direct effects and the production of anti-inflammatory SCFAs.

Pectins modulate microbiota-immune interactions in healthy mice

Since the DM and DB of pectins determine the direct effects of pectins on immunity, we also investigated how supplementation of these pectins influence microbiota-immune interactions under healthy conditions. Recent evidence demonstrated that the intestinal microbiota is very efficient in regulating intestinal T cells that are required for effective host defence against invading pathogens and avoidance of inflammation and disease [43]. Pectins can modulate microbiota communities [19,20] and influence T cell immunity in a microbiota dependent manner [44], but it is unknown how structural different pectins influence this interaction between microbiota and T cells. Hence, we investigated in **Chapter 5** the impact of DM and DB of pectins on the intestinal microbiota and T cell immunity after 1 and 4 weeks of pectin supplementation. The structural dependent effects of the pectins on T cell immunity in this chapter were different than the direct effects of the pectin structures on TLR2-1 as observed in **Chapter 3** and **4**. Here, one low DM pectin (DM19 (high DB)) and two intermediate DM pectins (DM49 (low DB) and DM43 (high DB)) increased levels of Th1, Th2 and Rorγt$^+$ Tregs in the mesenteric lymph nodes (MLNs), whereas another low DM pectin (DM18 (low DB)) did not influence T cell immunity. The differences in T cell stimulating effects between the pectin structures may derive from a differential impact of the pectins on the microbiota composition. The three pectins with T cell enhancing effects induced a relatively similar microbiota composition that was different from the DM18 (low DB) pectin supplemented mice. Such differences in microbiota composition may expose the intestinal immune system to a different set of microbial derived epitopes with immune enhancing effects such as lipopolysaccharide, or exopolysaccharides [45,46]. Additionally, the different T cell stimulating effects may also derive from a different composition of metabolic products. The T cell enhancing effects are not through enhancement of SCFA levels, as the pectin treatments did not increase SCFA levels, but may rather occur through the production of Ahr activating components. The caecal digesta from DM19 (high DB), DM49 (low DB), and DM43 (high DB) pectin supplemented mice did show higher Ahr activating properties, whereas caecal digesta from DM18 (low DB) pectin treated mice did not. The Ahr activation was present after 1 and 4 weeks of pectin supplementation, suggesting that Ahr activation may induce the effects on Rorγt$^+$ Tregs cells, and not on Th1 and Th2 cells, because these levels of these cells were dropped back to control levels after 4

weeks of pectin supplementation. Ahr activation might enhance CD103+ dendritic cell levels [47] which, together with CX3CR1+ macrophages, migrate to the MLN and enhance differentiation of CD4+ cells to Rorγt^{+} Tregs in the MLN [43]. Furthermore, Rorγt^{+} Tregs also express high levels of Ahr receptors [48], indicating that Rorγt^{+} Tregs expansion might result from the Ahr activation on these cells. Collectively, these studies suggest that the impact of pectins on the interaction between microbiota and T cells is dependent on specific DM and DB of pectins.

Although the 1 week and 4 week supplementation of the different pectin gives indications on structural dependent effects of pectins on the intestinal microbiota and T cell immunity over time, the conclusions are still based on correlative effects, and do not imply a causative effects of the pectin structures. Future mice studies using different doses may give more implications on whether the structures of the pectins are responsible for the effects on the intestinal microbiota and T cell immunity. Additionally, *in vitro* fermentation studies with the different pectin structures may also identify the specific microbial derived products with immune enhancing effects that are increased after pectin supplementation, such as the Ahr activating molecules. A fecal transplant of this digesta or postbiotic supplementation of these identified products to microbiota depleted mice[49], such as antibiotic treated mice [50] or germ free mice [51], may give more causative implications on the microbiota-dependent influence of the pectin structures on T cell immunity.

Pectins limit large intestinal inflammation through anti-pathogenic effects

Since the DM and DB of pectins induce anti-inflammatory effects through direct effect on intestinal immunity, we next investigated how such structural differences between pectins may induce anti-inflammatory effects through microbiota-dependent ways. Due to the high abundance of microbiota species in the large intestine (**Chapter 1**), we hypothesized that the microbiota-dependent effects of pectins on immunity to occur mostly in the large intestine. An infection with the enteric pathogen *Citrobacter rodentium* was used to study the microbiota dependent effect of the pectins as this pathogen specifically induce an altered microbiota composition that is characterized

by the overgrowth of this pathogen in the caecum and colon of mice [52]. Here, it induces inflammation of large intestinal tissues [53].

C. rodentium can penetrate through the inner and outer mucus layer of the large intestine [54], after which it forms attaching and effacing lesions on epithelial cells and induces epithelial barrier dysfunction and inflammation in the caecum and colon [53]. Since pectins were demonstrated to induce barrier protective effects on epithelial cells, we studied in **Chapter 6** how structurally different pectins protect from epithelial barrier disruption by *C. rodentium in vitro*. One low DM (DM32) pectin and two high DM pectins (DM59 and DM64) with a low or a high DB were used in this study to determine the DM and DB dependent effects. One low DM pectin was used in this study as the DB differences between low DM pectins can only be small as low DM pectins possess already a high number of non-esterified GalA residues. Furthermore, the DM64 with a high DB shows as similar strong TLR2-1 inhibition as the DM32 pectin (**Chapter 3**), whereas DM59 with a low DB does not, indicating that the DM and DB dependent effects of the pectins on TLR2-1 are comparable to the other used pectins. Our study demonstrated that pectins limited *C. rodentium* induced barrier disruption through antimicrobial effects. The protective anti-microbial effects of the pectins were independent of the DM and DB because all three pectins induced similar protective effects. The antimicrobial effects are suggested to occur through direct interaction of the homogalacturonan pectins with *C. rodentium* specifically. The exact mechanism through which pectins exert the antimicrobial effects should be further explored. Pectins are proposed to bind to surface molecules, such as lipopolysaccharides [12], or on a periplasmic pectin binding protein sph1118 that was identified in *Sphingomonas sp.* strain A1 as a binding receptor for pectin molecule[33]. Future studies identifying the specific surface molecules of *C. rodentium*, which are targeted by pectins, may be of importance for the design of pectin structures with high anti-microbial effects on *C. rodentium*.

In **Chapter 7**, the microbiota-dependent anti-inflammatory impact of structurally different pectins was investigated *in vivo* in mice with *C. rodentium* induced inflammation of the large intestine. Two high DM pectins (DM59 and DM64) that differed in DB were used in the current study. A higher dose of 5% of pectins was used in the current study as this pectin dose was previously shown to prevent experimental colitis [50]. This study showed that both pectins prevent the development

of *C. rodentium* induced colitis by protecting from *C. rodentium* induced epithelial disruption, intestinal damage and inflammation. Both low and high DB pectins protected against *C. rodentium*-induced damage in a similar way, independent of the DB. The pectins induced a relatively similar caecal microbiota composition, suggesting that the difference in DB does not induce differences in caecal microbiota compositions between the mice. Moreover, the protective effects of pectins were not derived from enhanced SCFA levels in het caecum, but rather from the lower bacterial load of *C. rodentium* in the caecum of pectin treated mice. These findings suggest that the distribution of methyl esters does not influence the beneficial effects of high DM pectins on *C. rodentium*-induced colitis in mice.

The pectins may prevent the *C. rodentium* overgrowth in these mice through several mechanisms. Firstly, both pectins stimulated the microbiota composition to a more rich and diverse composition compared to control mice treated with *C. rodentium* only, which was characterized by an increased abundance of beneficial bacteria. The enhanced stimulation of the growth of these microbes may prevent the overgrowth of *C. rodentium* in the caecum as they may compete for intestinal nutrients [55,56]. Secondly, pectins may also bind directly to *C. rodentium* and induce anti-microbial effects, as was found in **Chapter 6**. Thirdly, the pectins may be utilized by other intestinal microbes and digest pectins to GalA residues, which are known to inhibit the pathogenicity of *C. rodentium* through the interaction with the transcription factor ExuR [57]. In absence of GalA, the ExuR transcription factor stimulates the expression of the virulence genes of *C. rodentium* which leads to epithelial attachment and colitis. In presence of GalA, however, GalA binds to ExuR which suppresses the expression of specific virulence genes of *C. rodentium* [57]. Once *C. rodentium* loses virulence, it will not locate at the epithelium, but will localize to the lumen. Here, the growth of the pathogen can be outcompeted by other commensal bacteria, which lead to a lower *C. rodentium* load [55,56]. Future studies should investigate which through which mechanism pectins lower *C. rodentium* load as it may be of importance for the design of pectin structures that are easily digested by intestinal microbiota, or are rather indigestible to remain intact for anti-microbial effects. Studies with germ free mice with a *C. rodentium* infection that receive intact pectin molecules may determine whether the lower bacterial load is derived antimicrobial effects from microbial digestion of pectin molecules or from intact pectin molecules.

Conclusion and future perspectives

In this thesis, we determined how the DM and DB of homogalacturonan pectins influence direct effects and microbiota-dependent effects on immunity. Pectins with a low DM (~DM19) or pectins with an intermediate DM (~DM46) and a high DB are effective in inducing direct inhibitory effects on TLR2-1, whereas pectins with an intermediate DM and a low DB or high DM pectins (~DM86) are not. Through inhibition of TLR2-1, pectins can limit the development of small intestinal inflammation in mice with doxorubicin-induced mucositis. These findings suggest that the high number of blockwise distributed non-esterified GalA residues in pectins play a role in the direct effects on intestinal immunity. The microbiota-dependent effects on immunity are differently influenced by the DM and DB of homogalacturonan pectins. Low DM pectins with a high DB, and two intermediate DM pectins increased Th1, Th2 and Rorγt^{+} Tregs frequencies, whereas the low DM with a lower DB did not. Furthermore, we did not observe DM and DB dependent effects on anti-microbial effects on *C. rodentium* or DB dependent effects on protection in mice with *C. rodentium*-induced colitis. These findings suggest therefore that the use of homogalacturonan pectins with an intermediated DM and a high DB may influence immunity and enhance intestinal barrier function through both direct effects on immunity and microbiota-dependent effects.

This knowledge on homogalacturonan pectin structures can help in the application of pectins as functional foods and healthcare products. As mentioned before, our data suggest that the low DM pectins or intermediate DM pectins with a high DB pectins that inhibit TLR2 strongly may have anti-inflammatory effects on chemotherapeutically induced mucositis [31]. Additionally, consumption of different homogalacturonan pectin structures may also reduce the number of patients that are infected with the enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC). EPEC is still a common cause of diarrhea in low-income countries, whereas the EHEC remains a water-born pathogen in industrialized countries [58]. These pathogens have the same infection mechanism as *C. rodentium* [53], which virulence may be reduced after pectin consumption. Furthermore, humans with increased intestinal barrier dysfunction, such as patients with irriTable bowel syndrome [59] or inflammatory bowel diseases [60] could also benefit from the barrier protective effects of pectins.

In the design of functional foods or healthcare products, the dose and the duration of pectin treatment should be put into consideration as pectins have other functional properties, such as bulking effects, which may affect the beneficial effects of the pectin supplementation. Additionally, future studies should also distinguish between the prophylactic or therapeutic effect of pectins [9]. The studies in this thesis focused mostly on prophylactic effects, but pectins may be less effective when administered under inflammatory conditions [61]. Pre- and probiotic treatments were very efficient in reducing DSS-colitis in mice when administered prior to inflammation. However, the anti-inflammatory effects were minimal when these mice were administered with pre-and probiotics after the disease had been established [61]. The same may apply for pectin supplementation in IBD patients, which may have a disturbed food processing under inflammatory conditions. In these inflammatory phases of the disease, the fermentation of pectins may be disturbed and contribute to more inflammation in these patients [62]. Ultimately, the knowledge on the anti-inflammatory properties of pectins on intestinal immunity as described in this thesis can help the industry in tailoring pectin formulations with anti-inflammatory properties and can be used to treat immune-related disorders.

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