Impact of pectin dietary supplementation on experimental food allergy via gut microbiota modulation

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Other publications based on the results obtained in this project are currently in preparation.

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List of Abbreviations

Alum	Aluminium hydroxide
APC	Antigen-presenting cell
ASV	Amplicon sequence variant
AUC	Area under the curve
BCA	Bicinchoninic acid
CD	Cluster of differentiation
COX2	Cyclooxigenase-2
CT	Cholera toxin
CTL	Cytotoxic T cell
DB	Degree of blockiness
DC	Dendritic cell
DE	Degree of esterification
dH_2O	Distilled H_2O
FA	Food allergy
FACS	Fluorescence activated cell sorting
FcεRI	Fcε receptor I
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FOS	Fructo-oligosaccharides
GalA	Galacturonic acid
GOS	Galacto-oligosaccharides
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HG	Homogalacturonan
HMP	High methoxyl pectin
HRP	Horseradish peroxidase

huRBL	Humanized rat basophil leukemia cell
Ig	Immunoglobulin
i.g.	intragastric
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	intraperitoneal
ISAPP	International Scientific Association for Probiotics and Prebiotics
LMP	Low methoxyl pectin
LoD	Limit of detection
LP	Lamina propria
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
mMCPT-1	Murine mast cell protease-1
MW	Molecular weight
NF-κB	Nuclear factor kappa-B
nPru p 3	Natural Pru p 3
nsLTP	Non-specific lipid transfer protein
OAS	Oral allergy syndrome
OIT	Oral immunotherapy
OVA	Ovalbumine
PBS	Phosphate-buffered saline
PCoA	Principal component analysis
PE	Peach peel extract
PFA	Paraformaldehyde
PDG2	Prostaglandin 2
PRR	Pattern recognition receptors

RG-I	Rhamnogalacturonan-I
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
SEM	Standard error of means
sIgE/IgG/IgA	Specific IgE/IgG/IgA
SLIT	Sublingual immunotherapy
Th2 cell	T helper type 2 cell
TSLP	Thymic stromal lymphopoietin
TLR	Toll-like receptor
Treg	Regulatory T cell

1 Introduction

1.1 Food Allergy

Food allergies (FAs) are defined as adverse and reproducible immune reaction upon exposure to certain food antigens (Boyce et al. 2010; Gargano et al. 2021). Among the most frequent elicitors of FAs in adults are plant allergens, such as peanut, wheat, fruits, nuts and soybean, together with egg, milk and fish among others (Zuidmeer et al. 2008; Hefle et al. 1996; Maruyama 2021). Allergen exposure can induce a high variety of clinical symptoms in allergic individuals, including disorders of the gastrointestinal tract, airways or skin (van Splunter et al. 2020). In severe cases allergen ingestion might lead to cardiovascular aberrations, such as hypotension and life-threatening anaphylaxis (Mayorga et al. 2021). FAs are recognized as a growing public health as well as economic burden, with increasing prevalence in the recent decades (Warren et al. 2020). A population-based cross-sectional prevalence survey estimated that over 10% of the US population likely suffer from an immunoglobulin E (IgE)-mediated FA, affecting around 1 in 10 adults and 1 in 12 children (Gupta et al. 2019; Gupta et al. 2018). The annual healthcare system costs related to FAs were calculated around $24.8 billion in the US (Gupta et al. 2013). In Europe, the prevalence of FAs was reported to be 9.3% for children and 5.0% for adults (self-reported physician diagnosed) or 2.7% for children defined by clinical history or oral food challenge (Spolidoro et al. 2023). Among the most common food allergens that trigger allergic reactions were peanut, milk, fish, shrimp, celeriac, apple, peach and tree nuts with cow`s milk being the most common food allergen in early life (Sicherer et al. 2020; Fernández-Rivas et al. 2015).

Mechanistically, FAs can be distinguished in: IgE-mediated, non-IgE-mediated and a mixed form of IgE-dependent and IgE-independent, due to the immunological pathways (Yu et al. 2016; Wang and Sampson 2011). Most of the known non-IgE-mediated FAs exert subacute or chronic symptoms that are mainly located in the gastrointestinal tract but can also affect lung and skin (Zhang et al. 2021). Milk, soy, wheat and egg are common allergens inducing non-IgE-mediated FAs (Nowak-Węgrzyn et al. 2015). In contrast, IgE-mediated FAs are associated with a rapid onset of symptoms, such as urticaria, abdominal pain, wheezing and hypotension

but also severe or fatal anaphylactic reactions and therefore are the most detailed characterized type of FAs (Yu et al. 2016). The most common food allergens inducing IgE-mediated FA include e.g. peanut, milk, egg and shellfish whereas the prevalence of different FAs depends on the region and dietary habits as well (Yu et al. 2016; Hill et al. 2016; Lee et al. 2013). In addition, variants of IgE-mediated FAs exist, such as the oral allergy syndrome (OAS), characterized by specific IgE production against pollen-derived epitopes that show cross-reactivity with fruit- or plant-allergen epitopes (Ivković-Jureković 2015).

Despite the increasing number of patients suffering from FAs, no curative treatment is available so far. Different therapeutic options such as oral immunotherapy (OIT) or sublingual immunotherapy (SLIT), inducing complete or partial desensitization showed promising results in several studies (Fleischer et al. 2013; Burks et al. 2012; Vickery et al. 2018). However, these mentioned therapies are often not capable to induce complete tolerance. Currently the only approved product by the US Food and Drug Administration (FDA) and the European Commission for OIT is for treatment of peanut allergy (Bird et al. 2018; Moran and Burks 2015). In contrast, adverse reactions have been reported in patients upon OIT/SLIT that are commonly mild to moderate, such as symptoms of the gastrointestinal or respiratory tract but also development of severe anaphylactic reactions was reported (Anagnostou 2023; Vickery et al. 2018). Thus, to date the recommended management of FAs consists of avoidance of the allergenic food or symptomatic treatment including epinephrine, antihistamines or corticosteroids after accidental consumption (Muraro et al. 2022).

1.2 Mechanism of IgE-mediated food allergy

Immunologically, IgE-mediated FAs are typically caused by an allergic type I reaction (Figure 1), characterized by acute symptoms and a rapid onset within minutes to hours after exposure. This reaction is reproducible and occurs after each contact with the allergenic food and can be diagnosed by measurement of allergen-specific IgE (sIgE) (Tordesillas et al. 2017b; Ansotegui et al. 2020). Mechanistically, a sensitization phase upon the first contact with the allergen and an effector phase upon re-exposure to the allergen can be distinguished (Brough et al. 2020).

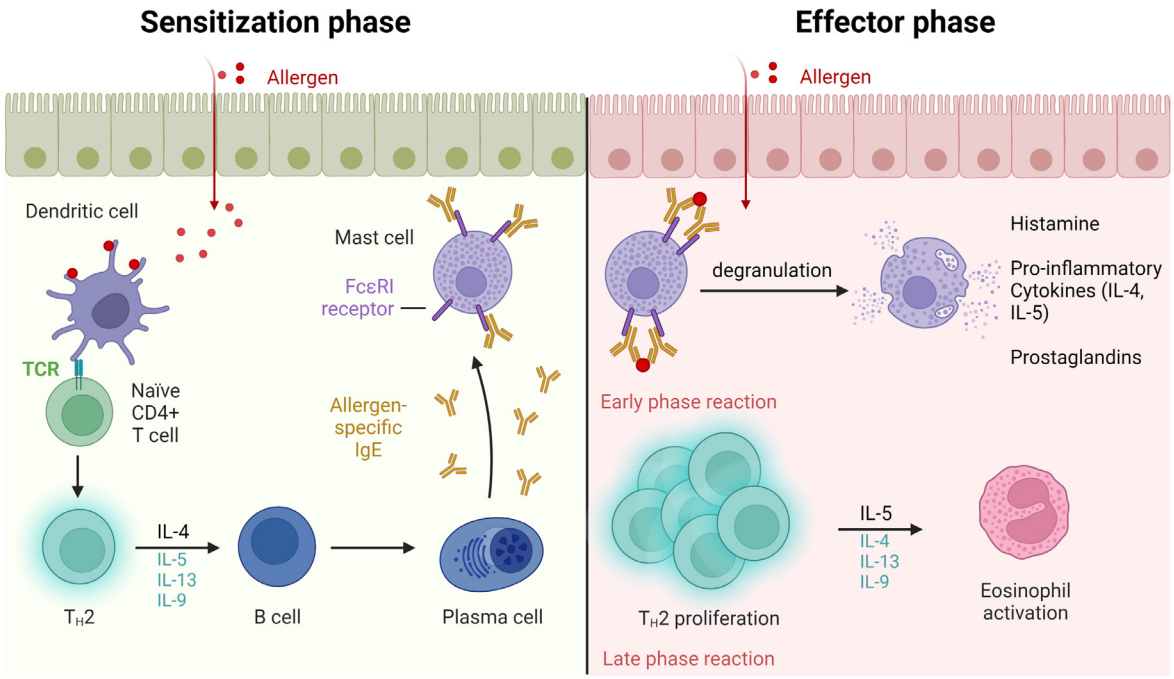

Figure 1: Mechanism type I allergy.
During the sensitization phase, food allergens are taken up by antigen-presenting cells (APCs), such as resident dendritic cells (DCs) and presented to naïve T cells. These cells differentiate into Th2 cells, releasing type-2 cytokines and promoting B cell differentiation into plasma cells that produce allergen-specific IgE (sIgE). sIgE binds to the Fcε receptor I (FcεRI) on mast cells and basophils and leads to sensitization of the cells. The effector phase is triggered by re-exposure to the specific allergen, leading to mast cell degranulation, release of different preformed mediators as e.g. histamine, and promotion of Th2 immune responses. Figure adapted from Mayorga *et al.* 2021.

During the sensitization phase, the food allergen passes across the epithelial barrier of skin, airways or gut, followed by uptake and internalization by specific mucosal-resident dendritic cells (DCs) (Ruiter and Shreffler 2012). The taken-up allergens are transported to draining lymph nodes, processed and presented to naïve $CD4^+$ T cells via major histocompatibility complex (MHC) class II molecules. This leads to differentiation of T cells into allergen-specific $CD4^+$ type 2 T helper (Th2) cells (Palomares et al. 2017). Th2 cells promote inflammatory signals by producing high levels of interleukin-4 (IL-4) and IL-13 and induce B cells to undergo class-switch to produce sIgE antibodies (Pulendran et al. 2010; Anvari et al. 2019). IgE is produced by plasma B cells in the lymph nodes but also locally in the intestinal tract or the airways, leading to the suggestion that IgE might be responsible for “organ-specific” allergic disorders (Coëffier et al. 2005; Galli and Tsai 2012). Secreted sIgE mainly binds to the high-affinity Fcε receptor I (FcεRI) expressed on the surface of mast

cells and basophils, inducing sensitization against the respective allergen (Palomares et al. 2017; Nagata and Suzuki 2022). In addition, epithelial cells contribute to allergen sensitization by production of pro-inflammatory cytokines, such as IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) that induce expansion and differentiation of DCs and thereby promote the Th2 response (Hammad and Lambrecht 2015; Divekar and Kita 2015).

The effector phase is triggered by re-exposure to the allergen, resulting in cross-linking of the FcεRI-bound sIgE and consequent aggregation of surface FcεRI (Zuurveld et al. 2022). This induces degranulation of mast cells and basophils and the release of different preformed mediators, that were stored in cytoplasmic granules, such as histamine, as well as newly formed mediators, as prostaglandin 2 (PDG2) or cytokines (Palomares et al. 2017). This so-called “immediate or early phase reaction” occurs within minutes after contact with the allergen and can lead to a variety of symptoms, including edema formation by increased vascular permeability, hypotension or even anaphylaxis (Galli and Tsai 2012). The following “late phase reaction” is characterized by accumulation of inflammatory mediators and activation of memory allergen-specific Th2 cells. Production of IL-4, IL-5, IL-9 and IL-13 leads to activation of eosinophils, maintenance of sIgE levels and recruitment of further inflammatory cells, such as basophils, neutrophils, monocytes and T cells to the tissue, prolonging the inflammatory processes and inducing tissue damage (Palomares et al. 2017; Galli et al. 2008; Kay 2001).

1.3 Non-specific lipid transfer proteins (nsLTPs)

1.3.1 The role of nsLTPs for allergic sensitization

A broad variety of plant food can induce allergic sensitization and, in the following, elicit allergy-related symptoms in patients. Especially allergens from the *Rosacea* family, including fruits from apple and/or peach are often involved in allergic reactions to plant-derived food (Fernández-Rivas et al. 2006). Mainly in the Mediterranean area but also in Northern and Central Europe as well as Asia, non-specific lipid transfer proteins (nsLTPs) are among the most important plant allergens and are associated with severe allergic symptoms (Javaloyes et al. 2012;

Scheurer et al. 2021). nsLTPs are small, non-glycosylated proteins of around 6 – 10 kDa that are highly resistant to heat and proteolytic digestion (Kader 1996). In plants they play an important role in the defense against biotic and abiotic stress and are involved in plant cytology, including stabilization of membranes, organization of cell walls and signal transduction (Liu et al. 2015). nsLTPs are structurally highly conserved panallergens that can frequently be found in terrestrial plants and pollen (Asero et al. 2001; Scheurer et al. 2021; Scheurer et al. 2004). To date, the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee has listed 52 nsLTPs (Kronfel et al. 2022). Depending on their molecular mass, two types of nsLTPs are distinguished: LTP1 (9 – 10 kDa; around 90 amino acids) and LTP2 (6 – 7 kDa; around 70 amino acids), whereas most of the allergenic nsLTPs belong to the first group (Lin et al. 2004; Skypala et al. 2021a).

Characterization of the crystal structure revealed a highly conserved structure of nsLTPs that consists of four alpha-helices, stabilized by four disulphide bridges and connected by flexible loops (Pasquato et al. 2006). Due to the high homology between different nsLTPs, cross-sensitizations are most common and patients sensitized to nsLTPs frequently develop allergic symptoms to a wide range of different vegetables and foods (Bogas et al. 2020; Egger et al. 2010). This so-called “nsLTP syndrome” may induce a variety of symptoms from mild contact urticaria, to OAS or gastrointestinal symptoms, up to severe anaphylactic reactions and plays an important role among the allergies to plants (Asero et al. 2002; Pascal et al. 2012).

1.3.2 nsLTP-mediated peach allergy

Peach allergy is one of the most frequent FAs and elicitor of anaphylactic reactions among adults in the Mediterranean area (Lyons et al. 2019; Schulten et al. 2009; Skypala et al. 2021a). Among the seven described peach allergens, the 9 kDa protein Pru p 3 (*Prunus persica*) is the only member of the nsLTP family with highest amounts present in the peach peel (Barni et al. 2022). Pru p 3 was described as primary sensitizer in many patients sensitized to nsLTPs and was therefore

identified as main marker allergen to assess nsLTP sensitization (Wolters et al. 2022; Asero et al. 2018). Sensitization to Pru p 3 can occur either as mono-sensitization or together with multiple nsLTP sensitizations, leading to nsLTP-syndrome and reactivity against several plant-food allergens (Skypala et al. 2021b). Studies could show that four major IgE epitopes of Pru p 3 are conserved among nsLTPs of fruits from the *Rosaceae* family (Borges et al. 2008; García-Casado et al. 2003), whereas cross-reactivity of nsLTPs also occurs between other plants, like vegetables and nuts (Schulten et al. 2011). Nevertheless, it was suggested that mono-sensitization goes along with more severe clinical reactions due to more efficient cross-linking of the IgE receptors (Scheurer et al. 2021). Characterization of Pru p 3 revealed 18 T-cell activating regions, inducing a Th2-dominated response, whereas in addition to the allergen itself, the natural ligands of nsLTP are suggested to play a role in allergenicity of the proteins due to an adjuvant-like effect (Schulten et al. 2009; Gonzalez-Klein et al. 2021). Studies identified members of the sphingolipid family as the main ligands of nsLTPs and the natural ligand of Pru p 3 could be identified as a derivate of camptothecin, bound to a phytosphingosine (Gonzalez-Klein et al. 2021; Cubells-Baeza et al. 2017).

1.4 Risk factors and prevention of food allergies

In a healthy state, the immune system can distinguish between pathogenic and environmental stimuli, leading to unresponsiveness and tolerance against common food antigens (Commins 2015). In food allergic individuals, this physiological distinction is inappropriate, resulting in break of tolerance and an inflammatory immune response upon ingestion of certain food allergens (Yu et al. 2016).

Multiple factors might play a role and promote the development of FAs. Studies suggest a role of genetic predisposition as well as epigenetic changes, whereas evidences are limited and FA prevalence is increasing faster than changes in the human genome might explain (Hong et al. 2016; Suaini et al. 2019; Peters et al. 2022). In addition, physical allergen characteristics as well as the time and route of sensitization seem to have an impact on the increasing prevalence of FAs (Sicherer and Sampson 2018). The “dual allergen exposure hypothesis”, based on the

observation that the majority of peanut allergics reacted on the first oral exposure, leads to the suggestion that the sensitization occurred non-orally but by exposure through the skin or respiratory tract, evading oral tolerance (Lack 2012; Kulis et al. 2021; Du Toit et al. 2016; Dębińska and Sozańska 2023). It was further found that several environmental factors, including air pollution (Brauer et al. 2007), pollen exposure (Susanto et al. 2022) or vitamin D insufficiency (Allen et al. 2013), might promote manifestation of FA.

Another explanatory approach for FA development are the microbial exposure and biodiversity hypotheses. These claim that absence of biodiverse environment or microbial exposure and infections in early life impact the development of the immune system and promote FAs (Markevych et al. 2017; Marrs et al. 2013). It is suggested that exposure to a biodiverse environment regulates the gut microbiome and promotes the development of a healthy immune system, whereas several factors that might promote FAs are associated with dysregulation of the microbiome (Pascal et al. 2018). Commensal bacteria on the skin or in the gastrointestinal tract influence the maturation of immune responses and studies revealed a shift towards Th2 responses as consequence of microbial environment aberration (Marrs et al. 2013). However, not only exposure to certain environment can shape the microbiota composition, also certain dietary fibers, especially prebiotics are known to modulate the microbiome. Studies revealed that proper fiber intake could optimize the microbiota composition leading to balanced Th1/Th2 immunity and reduction of allergic inflammatory responses (Zhang et al. 2016). Therefore, the potential of allergy prevention by supplementation of probiotics or prebiotics gained attention in recent years.

1.5 Dietary fibers

1.5.1 Definition and classification

Dietary fibers are defined as carbohydrates that are not hydrolyzed by endogenous human enzymes in the small intestine but can only be metabolized by certain species of gut microbiota by anaerobic fermentation (Cronin et al. 2021). Further differentiation regarding the non-carbohydrate components or the number of

monomers that constitute a fiber molecule are suggested. Here, definitions by national authorities differ, as in Europe a number of at least three monomers is required to define a polymer as fiber, whereas in other countries at least ten monomers might be required (Stephen et al. 2017; Menezes et al. 2013). Fibers consisting of a minimum of ten monomeric units are classified as *resistant starch* as well as *non-starch polysaccharides*, including e.g. cellulose, hemicellulose, inulin, β-glucan and pectin (O'Grady et al. 2019). In comparison, fibers consisting of three to nine monomeric units are entitled as *resistant oligosaccharides*, including galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (Eswaran et al. 2013). However, the specific characteristics and classification of different fibers as well as their effect on the host's health not only depend on the fiber structure but also on physiochemical characteristics, such as solubility and viscosity that also affect the fermentability by the host microbiome (Bijkerk et al. 2004). Insoluble fibers, such as cellulose are poorly fermented by the gut bacteria, whereas highly soluble and viscose fibers, as e.g. β-glucan or pectin are highly fermented in the gut (Holscher 2017). Furthermore, microbial fermentation of these fibers is promoted by their additional effect on glucose absorption and binding of bile acids (Holscher 2017). The location of fermentation of the dietary fibers depends on their degree of polymerization as well as the solubility. Soluble fibers, as FOS and pectin are mainly metabolized in the proximal gastrointestinal tract (ileum and ascending colon). Less soluble fibers, such as cellulose, can be partially fermented rather in the distal colon (Liu et al. 2016).

1.5.2 Prebiotics

Prebiotics were first defined in 1995 by Gibson and Roberfroid as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid 1995). However, this definition was further updated and revised according to advances in molecular methods (Gibson et al. 2010; Thomas et al. 2015). The current definition of a prebiotic was further elaborated by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "a substrate that is selectively utilized by host microorganisms conferring

a health benefit" (Gibson et al. 2017). Interestingly, not all dietary fibers are prebiotics, whereas most of the prebiotics can be classified as dietary fibers (Slavin 2013). To be classified as prebiotic three criteria are required (Gibson et al. 2004): 1) resistance to gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption; 2) fermentation by intestinal microbiota, and 3) selective stimulation of growth and/or activity of intestinal bacteria associated with health and wellbeing. In addition, prebiotics beneficially influence the health of the host by: 1) indirect increase of the secretion of bacteria-derived metabolites into the intestinal tract or by 2) direct effect on the immune response of certain cells, e.g. epithelial and immune cells (Brosseau et al. 2019).

The ability to ferment prebiotics highly varies between different bacteria. Several bacterial strains contain a high variety of enzymes to metabolize different complex carbohydrates, whereas others are only able to ferment a few polysaccharides (Onyango et al. 2021). Especially some microbiota of the genus of *Bacteroides* are able to metabolize a high range of different types of polysaccharides (Scott et al. 2014; Onyango et al. 2021). In general, the more complex a polysaccharide is, the more enzymes, such as glycosidases are necessary to metabolize it (Scott et al. 2014). The main bacterial end product of fermentation of complex carbohydrates are short chain fatty acids (SCFAs), such as acetate, propionate and butyrate (Markowiak-Kopeć and Śliżewska 2020; Vinelli et al. 2022; Koh et al. 2016). These could be shown to influence gastrointestinal epithelial cell integrity, glucose homeostasis, lipid metabolism, appetite regulation and immune functions (Blanco-Pérez et al. 2021; Martin-Gallausiaux et al. 2021).

1.6 Pectin

1.6.1 Definition and chemical structure

Pectin is a water-soluble dietary fiber that consists of a complex hetero-polysaccharide structure and can be found in cell walls and intercellular regions of higher plants (Chen et al. 2013). It is involved in a multitude of roles for plant development, such as connecting cell walls of neighboring plant cells or modulation of mechanical properties to regulate morphogenetic processes (Palin and Geitmann

2012). Due to its physicochemical characteristics, pectin is widely used as gelatinizer, thickener, stabilizer or fat replacer in food industry for the production of e.g. jams, yoghurt or ice cream (Willats et al. 2006). Pectins are mainly extracted from peel and pulp of different fruits or vegetables, such as apples, citrus fruits and sugar beets (Larsen et al. 2019). Most commonly, pectins are purified by acidic extraction followed by precipitation with ethanol, whereas the extraction method as well as the used source material can alter the chemical structure of the resulting pectins (Canteri-Schemin et al. 2005). The typical molecular mass of pectin is around 50,000 – 150,000 g/mol (Dongowski 1997).

In general, pectins consist of a linear backbone composed of homogalacturonan (HG) that contains at least 65% α-(1-4)-linked-D-galacturonic acid (GalA) units that can be acetylated at the C-2/C-3 hydroxyl groups or methyl-esterified at the C-6 carboxyl group (Dranca and Oroian 2018) (Figure 2).

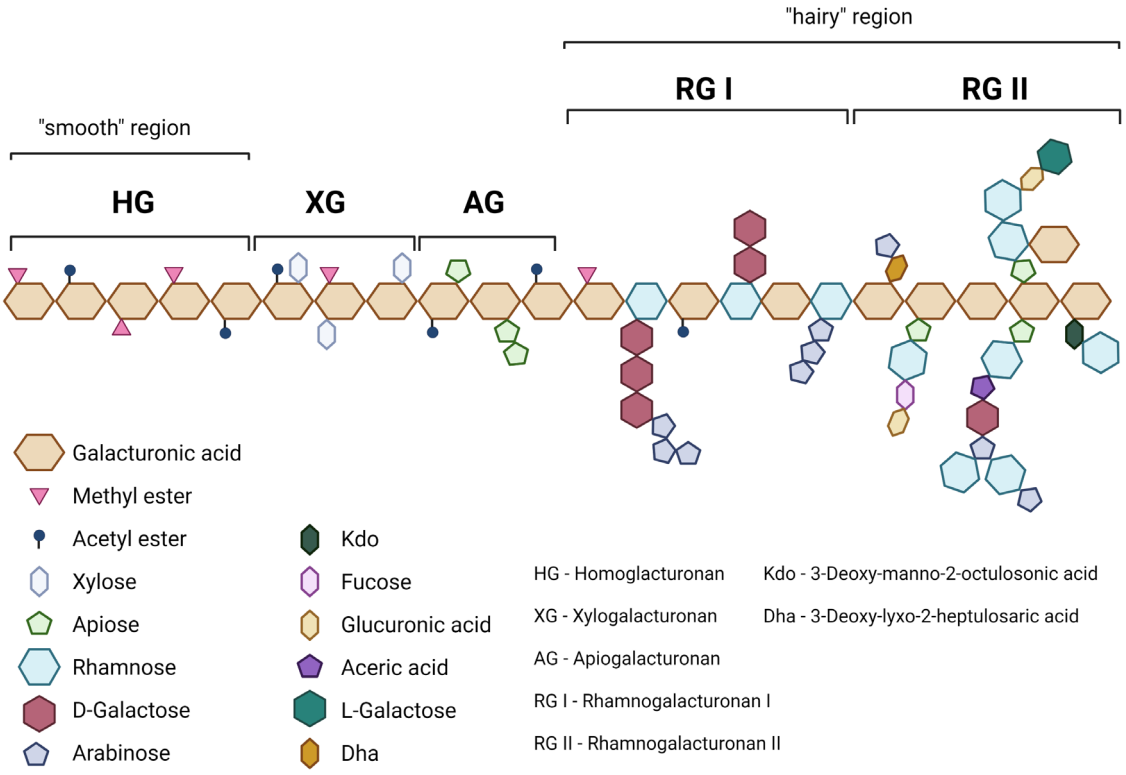

Figure 2: Schematic overview of pectin structure.
The chemical structure of pectin is highly variable. The backbone consists of galacturonic acid (GalA) units that can be methylated or acetylated. Depending on source material and extraction method, pectin molecules can contain different types and numbers of side chains and branches. Figure adapted from Beukema *et al.* 2020.

Different types of pectins can be classified by the ratio of esterification of GalA groups, termed as the degree of esterification (DE). Pectins with a DE > 50% are grouped as high methoxyl pectins (HMP) and pectins with a DE < 50% as low methoxyl pectins (LMP) (Liang et al. 2012; Wai et al. 2009). Most of the natural, non-processed pectin is classified as HMP with a DE of ~ 80%, whereas most of the pectin in processed foods is classified as LMP (Sila et al. 2009; Vanitha and Khan 2020). Next to the linear backbone, branched ("hairy") regions like rhamnogalacturonan-I (RG-I) can occur, providing attached neutral sugars, e.g. galactan, arabinan or arabinogalactans (Sila et al. 2009).

Pectins are classified as prebiotics, which are not digestible by human enzymes but can be degraded in the intestinal tract by commensal bacteria, promoting the growth and colonization of different bacterial strains, especially *Bifidobacteria*, *Lactobacilli* or *Bacteroides* species (Tingirikari 2018; Gómez et al. 2014). By this, pectin consumption may positively affect homeostasis of the gut microbiome (Hasan and Yang 2019). Furthermore, two health claims have been granted for pectins in the EU: 1) reduction of the blood glucose rise after meals and 2) maintenance of normal blood cholesterol levels after consumption of at least 6 - 10 g pectin per meal (EFSA Panel on Dietetic Products, Nutrition and Allergies 2010).

Different fermentation efficiency could be observed due to structural characteristics of the pectin type, as LMP was shown to be fermented faster than HMP (Dongowski et al. 2002). Furthermore, it has been shown that the backbones of pectin macromolecules might exert immunosuppressive activities (Popov and Ovodov 2013). Thus, the structural features of pectin such as DE, molecular weight (MW) and distribution of side chains as well as free carboxylic groups might therefore also determine the immune-modulatory properties of pectin (Blanco-Pérez et al. 2021; Hino et al. 2013). Nevertheless, knowledge of the effect of distinct pectin structures in regard of the DE, MW and side chains on the cellular and humoral immune response is limited.

1.6.2 Immune-modulatory effects of pectin

Pectins provide different immune-modulatory properties that can be either achieved by direct effects on immune cells or indirectly mediated by bacterial metabolites upon fermentation in the gut (Larsen et al. 2019) (Figure 3). Pectins can directly interact with intestinal and mucosal cells, promoting immune cell responses by interaction with pattern recognition receptors (PRRs) (Prado et al. 2020). Different immune-modulatory effects might thereby be caused by different pectin binding characteristics (Beukema et al. 2020). It was shown *in vitro*, that especially LMP binds to the ectodomain of Toll-like receptor 2 (TLR2) by electrostatic interactions and specifically inhibits the pro-inflammatory TLR2-TLR1 pathway (Sahasrabudhe et al. 2018). The immune-modulatory effects of pectin mainly depend on the content of GalA residues and DE (Chen et al. 2006). It could be shown that HMP inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) expression in murine peritoneal macrophages. Furthermore, HMP inhibited MAPK phosphorylation, IKK kinase activity and NF-κB activation more efficiently than LMP (Chen et al. 2006).

Indirect effects of pectins are mediated via fermentation by commensal gut microbiota and production of beneficial SCFAs (van der Beek et al. 2017), whereas fermentation of structurally different pectins generates distinct profiles of SCFAs (Larsen et al. 2019). Generation of SCFAs can promote anti-inflammatory effects, such as enhanced generation of colonic regulatory T cells (Tregs) (Smith et al. 2013), inhibition of Th2-mediated airway diseases shown in mice (Canani et al. 2011), stimulation of epithelial cell growth and suppression of the activation of antigen-presenting cells (APCs) (Macfarlane and Macfarlane 2012). These immune-modulations by SCFAs are caused by three different mechanisms: 1) direct activation of G-coupled receptors, 2) induction of epigenetic modifications by inhibition of histone deacetylase (HDAC) and 3) serving as energy substrate for immune and non-immune cells (Watt et al. 2020; Tan et al. 2014).

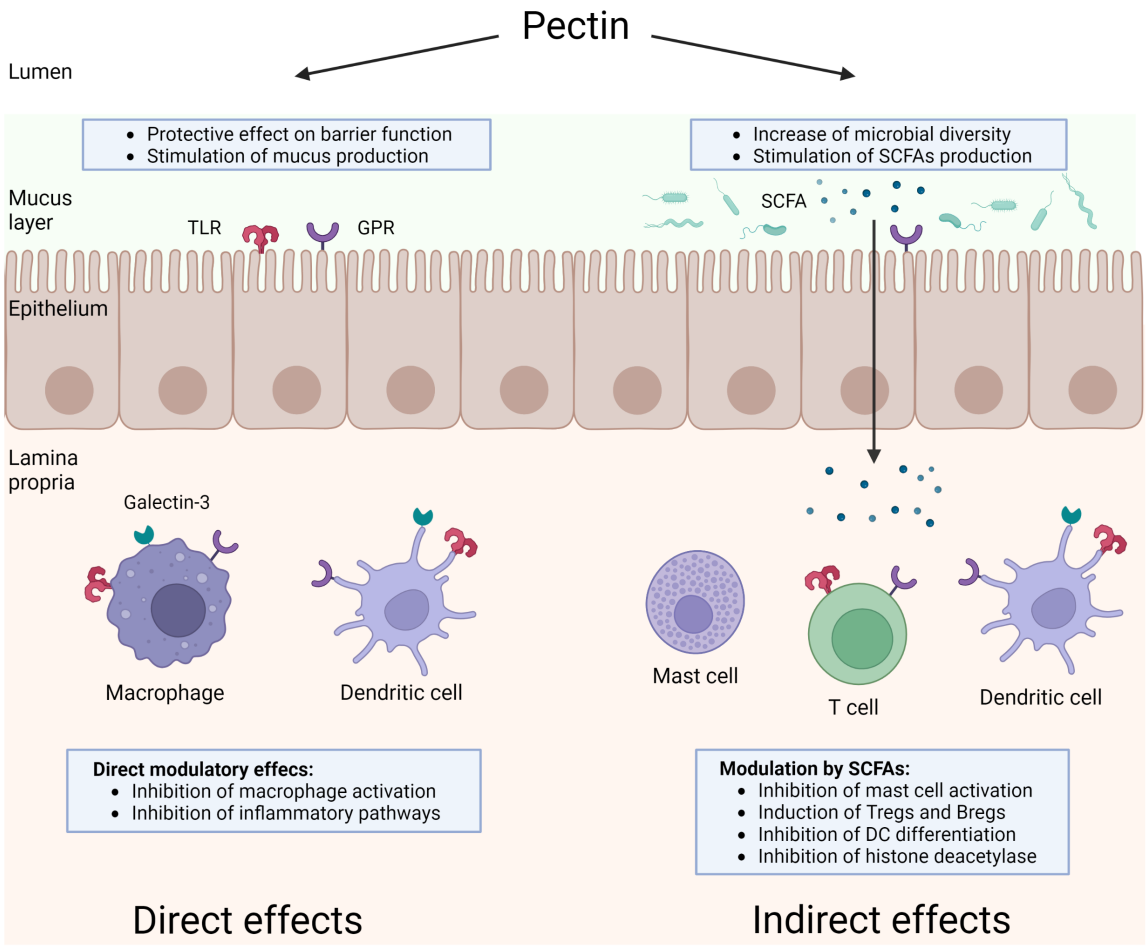

Figure 3: Direct and indirect immune-modulatory effects mediated by pectin.
Pectins can exert direct effects on immune cells or epithelial cells by interaction with Toll-like receptors (TLRs) or G-protein coupled receptors (GPRs). By modulation of the microbiota composition and production of short chain fatty acids (SCFAs) as fermentation product, pectin can also exert indirect immune-modulatory effects. Figure adapted from Beukema *et al.* 2020.

1.6.3 Pectin effects on allergy

The role of pectins in the manifestation of allergies is controversially discussed. Several studies reported either a promoting or a preventive effect of different pectins on allergic diseases (Ferdman et al. 2006; Capucilli et al. 2019; Iwamoto et al. 2019; Räsänen et al. 1998). Due to matrix effects, pectin was shown to hamper digestibility of food allergens by pepsin *in vitro*, or to reduce the accessibility of allergen cleavage-sites, both promoting the process of allergenic sensitization (Polovic et al. 2007; Peyron et al. 2006). Furthermore, it could be shown that oral administration of citrus pectin inhibited induction of oral tolerance after feeding mice with a high

dose of ovalbumin (OVA) (Khramova et al. 2009). In addition, allergic reactions upon pectin exposure have been reported. Reports from the 1990s suggest that continuous airborne exposure to pectin at the work place could be associated with the development of occupational asthma (Jaakkola et al. 1997; Kraut et al. 1992). In a further case, allergic reactions were reported after ingestion of pectin-containing food (Capucilli et al. 2019; Uno et al. 2017).

In contrast, several beneficial effects of pectin on allergic sensitization were reported (Popov and Ovodov 2013; Iwamoto et al. 2019; Lee et al. 2004; Jang et al. 2021; Trompette et al. 2014). A study revealed that pectins containing more than 80% GalA residues were able to suppress macrophage activity and furthermore inhibit delayed-type hypersensitivity reaction (Popov and Ovodov 2013). Additionally, it was found that alkali-soluble pectin suppressed IgE production in human myeloma cell lines (Iwamoto et al. 2019). Interestingly, high-pectin diets could also suppress inflammatory responses as well as development of allergic asthma in mice, which is suggested to be mediated by modulation of the microbiome and changes in the production of certain immune-modulatory metabolites (Lee et al. 2004; Jang et al. 2021; Trompette et al. 2014). However, whether pectin exerts beneficial effects on FAs and if structurally different pectins induce distinct immune responses is still not clarified.

2 Working hypothesis and objectives

In recent years, dietary fibers gained focus in regard of their immune-modulatory effects and the potentially beneficial effect on allergies. The dietary fiber and prebiotic pectin is able to promote growth and activity of beneficial bacteria and thereby induce modulation of different immune responses. However, structurally different types of pectin might promote different immune-modulatory responses and to date the optimal pectin type for induction of beneficial health effects is not identified. Furthermore, it is still unclear, whether pectins provide a beneficial effect on certain allergies, such as FA.

Having this in consideration, the following working hypotheses were set up:

1) Structurally different types of pectins exert certain immune-modulatory effects.
2) Pectins exert a beneficial effect on FAs due to gut microbiota regulation.

To evaluate the effect of pectin on FA, peach allergy was used as model for severe FA. This project was part of an international consortium (BMBF-ERA 01EA1901), with a human dietary intervention being performed in parallel. The here described study focused on the mechanistic examination in an experimental mouse model.

To examine the hypotheses, the following experimental objectives were set up:

1) Characterization of two different commercial pectins in regard of residual nsLTP content.
2) Examination of immune-modulatory effects of structurally different pectins on naïve mice.
3) Establishment of a peach allergy mouse model.
4) Dietary intervention with pectin in peach allergic mice.

Work program:

As in the human dietary intervention which was part of the collaborative project, peach allergics should consume a high amount of pectin, residual nsLTP in pectin and a potential nsLTP-cross-reactivity had to be excluded for safety reasons. Therefore, in this study the two different pectins were characterized in regard to putative amounts of residual nsLTP. In a next step, the immune-modulatory effect of different pectins supplemented in different amounts to the food was examined in naïve mice. The effect of the pectin supplementation was furthermore evaluated in three mouse strains (CBA/J, C57BL/6 and BALB/c). This objective revealed the optimal amount of dietary pectin supplemented to the mice for further use in the allergy intervention study.

To study the effect of pectin on peach allergy, a peach allergy mouse model was established. To achieve this, different sensitization and provocation protocols were tested in regard to a homogeneous development of clinical signs as well as antigen-specific IgE levels. In addition, peach protein extraction was established and used for sensitization and provocation. In the final established peach allergy mouse model, the effect of dietary pectin intervention was examined.

The results obtained in this study should provide evidence whether pectins exert beneficial effects on peach allergy and if structurally different types of pectins induce certain immune-modulatory effects. This study should therefore contribute to gain better understanding of the effects of prebiotics on microbiota composition and subsequently on immune-response in healthy as well as food allergic mice.

3 Material and Methods

3.1 Material

3.1.1 Pectin

Commercial pectins from different source materials, containing different DE and GalA content were provided by Herbstreith & Fox KG, Neuenbürg, Germany (Table 1).

Table 1: Provided pectins from different source material and characteristics.

Name	Source	DE	GalA	Classification
Classic CU901	Citrus	7.3%	91%	LMP
Herbapekt SF 50-LV	Apple	57%	82%	HMP

For the study, two pectins were chosen according to their DE and solubility in distilled H_2O (dH_2O) or pectinase solution (Figure S1): apple-derived high methoxyl pectin (HMP) Herbapekt SF 50-LV (DE of 57%, 82% GalA and low MW) and the citrus-derived low methoxyl pectin (LMP) Classic CU901 (DE of 7.3%, 91% GalA and low MW).

3.1.2 Peaches

Fresh ripe peaches (Royal summer® variety) were obtained from a local grocery store peeled and the peel was directly frozen at -80°C until further use.

3.1.3 Pectin containing diet

Food pellets for dietary pectin supplementation in mouse experiments including 20% cellulose, 5% pectin + 15% cellulose or 15% pectin + 5% cellulose using either HMP Herbapekt or LMP CU901 were prepared by ssniff Spezialdiäten GmbH (Soest, Germany) according Table 2.

Table 2: Food pellet composition for pectin supplementation.

Ingredient	U	20 % Cellulose AIN93G mod. S8144-E730	5 % Pectin 15% Cellul. AIN93G S8144-E732 / E736	15 % Pectin 5% Cellul. AIN93G S8144-E734 / E738
Casein	%	20.0000	20.0000	20.0000
L-Cystine	%	0.3000	0.3000	0.3000
Cellulose powder	%	20.0000	15.0000	5.0000
Pectin [1)2)]	%	---	5.0000	15.0000
Corn starch	%	26.7486	26.7486	26.7486
Maltodextrin	%	13.2000	13.2000	13.2000
Sucrose	%	10.0000	10.0000	10.0000
Vitamin premix	%	1.0000	1.0000	1.0000
Mineral premix	%	3.5000	3.5000	3.5000
tBHQ	%	0.0014	0.0014	0.0014
Choline bitartrate	%	0.2500	0.2500	0.2500
Soybean oil	%	5.0000	5.0000	5.0000
Proximate contents				
Crude protein	%	17.6	17.6	17.6
Crude fat	%	5.1	5.1	5.1
Crude fiber	%	19.9	19.3	19.1
Crude ash	%	3.2	3.2	3.2
Starch	%	25.7	25.7	25.7
Dextrin	%	13.0	13.0	13.0
Sugar	%	11.2	11.2	11.2
Energy (Atwater) [3)] MJ/kg		**13.4**	**13.4**	**13.4**
kcal% Protein		22	22	22
kcal% Fat		15	15	15
kcal% Carbohydrates		63	63	63

[1)] Calculated with 94 % fiber
[2)] Pectin provided by the customer: CU 901 and Herbapekt
[3)] = Physiological fuel value

3.1.4 Equipment and instruments

The used equipment is listed in the following Table 3.

Table 3: Equipment and Instruments.

Device	Model	Manufacturer
Autoclave	DX-65	Systec GmbH, Linden, Germany
Balances	LE2202S Explorer Pro Advanced LPWG 723I	Sartorius, Göttingen, Germany Ohaus, Nänikon, Switzerland VWR, Darmstadt, Germany
Centrifuges	Micro Centrifuge 100 Vac Centrifuge 5417 R Megafuge 1.0R Multifuge 1S-R Sorvall Lynx 4000	Carl Roth, Karlsruhe, Germany Eppendorf, Hamburg, Germany Heraeus, Hanau, Germany Heraeus, Hanau, Germany Thermo Fisher Scientific, Darmstadt, Germany
Cation exchange column	HiPrep™ SP HP 16/10	Cytiva, Munich, Germany
CO_2 cell incubator	BBD 6220	Heraeus, Hanau, Germany
Electrophoresis power supply	Power Pack P25	Biometra, Jena, Germany
ELISA reader	SpectraMax Plus 340	Molecular Devices, Munich, Germany
ELISA washer	ELX405	Bio-Tex, Neufahrn, Germany
Flow cytometry	BD FACS LSRII SORP Symphony	BD Biosciences, Heidelberg, Germany
Fluorometer	Quantus™	Promega, Walldorf, Germany

Device	Model	Manufacturer
Food processor	BioHomogenizer M133/2280	Biospec Products, Bartlesville, USA
FPLC	ÄKTA pure	GE HealthCare, Munich, Germany
Gel electrophoresis chamber	Mini-PROTEAN® 3 cell	Bio-Rad, Dreieich, Germany
Grinder	Grindomix GM200	Retsch GmbH, Haan, Germany
iBright	FL1500	Thermo Fisher Scientific, Darmstadt, Germany
Laminar air flow	SterilGARD III	Labotect, Rosdorf, Germany
Magnetic stirring and heating plate	MR Hei-Tec	Heidolph Instruments, Schwalbach, Germany
Microprobe thermometer	BAT-12	Physitemp Instruments LLC, New Jersey, USA
Microscopes	ID 03	Zeiss, Roßdorf, Germany
QIACube	QIAcube Connect	Qiagen, Hilden, Germany
Size exclusion column	HiPrep™ 26/60 Sephacryl S-100 HR column	Cytiva, Munich, Germany
Thermocycler	Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortex	REAX 2000	Heidolph Instruments, Schwalbach, GErmany
Water bath	1003	GFL, Burgwedel, Germany

3.1.5 Chemicals

The used chemicals are indicated in Table 4.

Table 4: Used chemicals.

Reagent / chemical	Manufacturer
Acetone	Merck KgaA, Darmstadt, Germany
Ammonium bicarbonate	Sigma-Aldrich, Munich, Germany
Bicinchoninic acid assay (BCA)	Thermo Fisher Scientific, Darmstadt, Germany
Bovine serum albumin (BSA)	Carl Roth, Karlsruhe, Germany
Bromphenol Blue sodium salt	Sigma-Aldrich, Munich, Germany
Calcium chloride ($CaCl_2$)	Carl Roth, Karlsruhe, Germany
Cholera toxin	Sigma-Aldrich, Munich, Germany
Collagenase D from *Clostridium histolyticum*	Sigma-Aldrich, Munich, Germany
Di-Sodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, Munich, Germany
Dispase II protease	Sigma-Aldrich, Munich, Germany
Dithiothreitol (DTT)	AppliChem GmbH, Darmstadt, Germany
Sodium diethyldithiocarbamate trihydrate (DIECA)	Carl Roth, Karlsruhe, Germany
Dnase I grade II, from bovine pancreas	Sigma-Aldrich, Munich, Germany
Ethanol 70% (v/v)	Merck KGaA, Darmstadt, Germany

Reagent / chemical	Manufacturer
Fetal Calf Serum (FCS)	Sigma-Aldrich, Munich, Germany
FoxP3/Transcription factor staining set	Thermo Fisher Scientific, Darmstadt, Germany
Fructozym® P6-XL (EC. 3.2.1.15)	Erbslöh Geisenheim GmbH, Geisenheim, Germany
GelCode Blue	Thermo Fisher Scientific, Darmstadt, Germany
Glycerine	Carl Roth, Karlsruhe, Germany
Histofix (phosphate buffered, 4%)	Carl Roth, Karlsruhe, Germany
Imject® Alum (aluminium hydroxide and magnesium hydroxide)	Thermo Fisher Scientific, Darmstadt, Germany
Ionomycin	Sigma-Aldrich, Munich, Germany
Methanol	Merck KGaA, Darmstadt, Germany
NucleoSpin DNA Stool Kit	Macherey-Nagel, Düren, Germany
Nuclear staining kit (true nuclear)	BioLegend, Fell, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, Munich, Germany
Phorbol-12-myristat-13-acetat (PMA)	Sigma-Aldrich, Munich, Germany
Potassium chloride (KCl)	Sigma-Aldrich, Munich, Germany
Potassium di-hydrogen phosphate (KH_2PO_4)	Carl Roth, Karlsruhe, Germany
Protease inhibitor cocktail set 1	Merck, Darmstadt, Germany

Reagent / chemical	Manufacturer
Polyvinylpyrrolidone (PVPP)	Merck, Darmstadt, Germany
QIAamp PowerFecal Pro DNA Kit	Qiagen, Hilden, Germany
QuantiFluor ONE dsDNA System Kit	Qiagen, Hilden, Germany
Qubit dsDNA HS assay	Thermo Fisher Scientific, Darmstadt, Germany
Rotiphorese Gel 30	Carl Roth, Karlsruhe, Germany
Roti Quant	Carl Roth, Karlsruhe, Germany
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich, Munich, Germany
Sodium acetate (NaOAc)	Carl Roth, Karlsruhe, Germany
Sodium azide (NaN_3)	Sigma-Aldrich, Munich, Germany
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich, Munich, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, Munich, Germany
Sodium di-hydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, Munich, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich, Munich, Germany
Sulfuric acid (H_2SO_4) 5N	Merck, Darmstadt, Germany
Tris	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Munich, Germany
Trypan Blue	Sigma-Aldrich, Munich, Germany
Tween® 20	Sigma-Aldrich, Munich, Germany

Reagent / chemical	Manufacturer
Lysing Buffer (red blood cell lysis buffer)	BD Biosciences Pharm Lyse™, Heidelberg, Germany
2-mercaptoethanol	Sigma-Aldrich, Munich, Germany
3, 3', 5, 5'-tetramethylbenzidine (TMB)	Carl Roth, Karlsruhe, Germany
6-aminohexanoic acid	Carl Roth, Karlsruhe, Germany

3.1.6 Buffers, solutions and media

Table 5: Solutions and buffers used.

Solution / Buffer	Usage	Ingredients or manufacturer
Ammonium bicarbonate buffer (0.05 M)	Peach protein extraction, n Pru p 3 purification	2% PVPP, 2 mM EDTA, 10 mM DIECA, 100 mM $CaCl_2$ in 0.05 M ammonium bicarbonate; pH 8.3
Blocking buffer ELISA	ELISA	10% FCS in PBS
Blocking buffer immunoblot	Immunoblot	0.3 % Tween in TBS
BSA solution	Standard for BCA and Bradford, Inhibition-Immunoblot	1-3 % BSA in PBS
Coating buffer (50 mM)	ELISA	16 mM Na_2CO_3, 34 mM $NaHCO_3$ in 1 L ddH_2O; pH 9.6

Solution / Buffer	Usage	Ingredients or manufacturer
Digestion solution	Lamina propria dissociation	0.5 mg/ml Collagenase D, 0.5 mg/ml DNase I, 1 mg/ml Dispase II in PBS
EDTA	Lamina propria dissociation	0.5 M disodium EDTA in H_2O; pH 7.5
FACS buffer	FACS	1 % BSA, 20 mM EDTA, 0.03% NaN_3 in PBS
Fixation buffer	FACS	1% PFA in PBS
Hanks' Balanced Salt Solution (HBSS)	Lamina propria dissociation	Gibco, Thermo Fisher Scientific, Darmstadt, Germany
HEPES buffer	Lamina propria dissociation	1 M 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethane sulphonic acid; pH 7.9
Lysis buffer	Lamina propria dissociation	3 mg/ml collagenase 1A in DMEM
MACS buffer	Lamina propria dissociation	5% bovine serum albumin (BSA), 2mM ethylenediaminetetraacetic acid (EDTA) in PBS
Phosphate buffered saline (PBS)	ELISA, cell culture, Lamina propria dissociation	1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 3 mM KCl in H_2O; pH 7.1
Pre-digestion solution	Lamina propria	1xHBSS, 5 mM EDTA, 10 mM HEPES

Solution / Buffer	Usage	Ingredients or manufacturer
TMB substrate	ELISA	0.525 mM TMB, 0.01% H_2O_2 in 0.21 M potassium citrate buffer; pH 3.95
Tris buffered saline (TBS)-Tween 0.05%	Immunoblot	20 mM Tris, 150 mM NaCl, 0.05% Tween® 20 (w/v) in H_2O
Trypan Blue solution	Cell counting	4% Trypan Blue in PBS
Washing buffer	ELISA	0.05% Tween® 20 in PBS
RPMI 1640	Splenocyte/MLN stimulation	Gibco by life technologies, USA
Modified Eagle's minimal essential medium (MEM)	Cell culture	Gibco by life technologies, USA

3.1.7 Antibodies

The antibodies that were used for flow cytometric analysis are listed in Table 6, including fluorophore, clone and manufacturer.

Table 6: Antibodies used for FACS analysis.

Target	Label	Clone	Manufacturer
CD3	BV421	17A2	BioLegend, Fell, Germany
CD4	PECy7	GK1.5	BioLegend, Fell, Germany
CD8a	BF510	53-6.7	BioLegend, Fell, Germany
CD11b	BV421	M1/70	BioLegend, Fell, Germany
CD11c	BV605	N418	BioLegend, Fell, Germany

Target	Label	Clone	Manufacturer
CD19	PE-BV605	6D5	BioLegend, Fell, Germany
CD25	BV711	PC61	BioLegend, Fell, Germany
CD45	FITC	30-F11	BioLegend, Fell, Germany
CD64	PECy7	X54-5/7.1	BioLegend, Fell, Germany
CD117	BV711	2B8	BioLegend, Fell, Germany
FoxP3	PE	150D	BioLegend, Fell, Germany
Ly6G	Alexa647	1A8	BioLegend, Fell, Germany
MHCII	BV510	M5/114.15.2	BioLegend, Fell, Germany
Siglec-F	PE	S17007L	BioLegend, Fell, Germany
CD16/32	-	93	eBioscience, Frankfurt, Germany
Viability dye	eFluor™ 450	-	Thermo Fisher, Darmstadt, Germany

The following Table 7 lists the antibodies used for ELISA.

Table 7: Antibodies used for ELISA.

Antibodies	Clone	Manufacturer
Purified anti-mouse IgE	R35-72	BD Biosciences, Heidelberg, Germany
Biotin Anti-mouse IgE	RE35-118	BD Biosciences, Heidelberg, Germany

Antibodies	Clone	Manufacturer
Anti-human/mouse IL-5 purified	TRFK5	ThermoFisher Scientific, Darmstadt, Germany
Biotin Anti-mouse IL-5	TRFK4	eBioscience, Frankfurt, Germany
Anti-mouse IFNγ purified	XMG1.2	eBioscience, Frankfurt, Germany
Biotin Anti-mouse IFNγ	R4-6A2	eBioscience, Frankfurt, Germany
Anti-mouse IgG1 ($_{\gamma}$1) horseradish peroxidase (HRP conjugate)	-	ThermoFisher Scientific, Darmstadt, Germany
Anti-mouse IgG2a horseradish peroxidase (HRP conjugate)	-	ThermoFisher Scientific, Darmstadt, Germany
mMCPT-1 mouse uncoated ELISA Kit	-	ThermoFisher Scientific, Darmstadt, Germany
IL-4 kit	-	R&D Systems, Wiesbaden, Germany
Ready set GO! Anti-mouse IL-13	-	ThermoFisher Scientific, Darmstadt, Germany
Ready set GO! Anti-mouse IL-33	-	ThermoFisher Scientific, Darmstadt, Germany
Ready set GO! Anti-mouse total IgG	-	ThermoFisher Scientific, Darmstadt, Germany
Ready set GO! Anti-mouse total IgA	-	ThermoFisher Scientific, Darmstadt, Germany

Antibodies	Clone	Manufacturer
Ready set GO! Anti-mouse total IgE	-	ThermoFisher Scientific, Darmstadt, Germany
Streptavidin-HRP	-	BD-Pharmingen

In the following Table 8 the antibodies and sera used for immunoblot and mediator release assay with humanized rat basophil leukemia cells (huRBLs) are listed.

Table 8: Antibodies and sera used for immunoblot and huRBL assay.

Antibodies	Species	Manufacturer
anti-Pru av 3 serum	rabbit	CE-Immundiagnostika
pre-immune serum	rabbit	CE-Immundiagnostika
anti-rabbit IgG (HRP-conjugate)	goat	Cell Signaling
anti-Peach/Pru p 3 serum	mouse	PEI (own experiments)
anti-mouse IgG1 (AP-conjugate)	rat	BD Pharmingen
Pru p 3-reactive patient serum	human	Provided by Dr. Anna Cistero-Bahima (Universitari Dexeus of Barcelona, Spain)

3.1.8 Cell lines

The cell line huRBL-30/25 as described by Vogel *et al.* was used in this study for β-hexosaminidase release (Vogel et al. 2006). The assay is described in detail in chapter 3.2.17.

3.1.9 Software

The software used for data analysis and visualization is listed in Table 9. For preparation of images, biorender.com was used.

Table 9: Used Software.

Software	Manufacturer
Citavi Version 6	Swiss Academic Software GmbH, Switzerland
FACSDiva™ Version 6.1.3	BD Bioscience, Heidelberg, Germany
FlowJo Version 10.0.8r1	FlowJo LLC, Oregon, USA
GraphPad Prism 9.2.0	GraphPad Software, Inc., USA
Image J Version 1.53C	LOCI, University of Wisconsin, USA
MS Office 2019	Microsoft Deutschland GmbH, Germany
Qiime 2	Qiime 2 development team
R studio v.1.22.0	Rstudio Inc., Boston, USA
SoftMax®Pro Software, Version 5.2 rev C	Molecular Devices, Munich, Germany

3.2 Methods

3.2.1 Animals

Female CBA/J, C57BL/6 and BALB/c mice were purchased from Charles River Deutschland GmbH, and housed under specific pathogen-free conditions in the animal facility of the Paul-Ehrlich-Institut with free access to water and food. All animal experiments were performed in compliance with the German animal protection law (granting authority: RP Darmstadt, Germany, Approval numbers: F107/2003 and F107/2005). Mice were 6 – 8 weeks old when the experiment started and were randomly assigned to the experimental groups.

3.2.2 Dietary pectin supplementation to naïve mice

Female CBA/J, C57BL/6 and BALB/c mice (n = 8 per group; 2 mice per cage) were fed *ad libitum* a control diet (20% cellulose) for 14 days, followed by 14 days feeding of the respective pectin diet (5% or 15% HMP or LMP) (Figure 4).

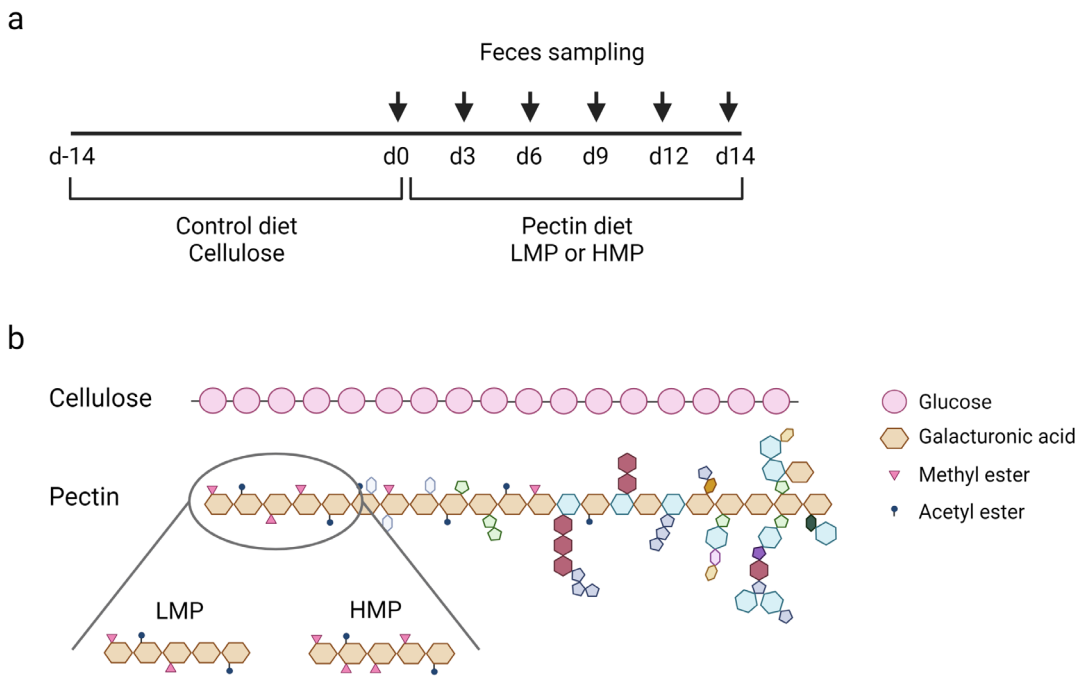

Figure 4: Pectin supplementation schedule.
(a) For the pectin supplementation experiment naïve CBA/J, C57BL/6 and BALB/c mice were fed a control diet for 14 days, followed by the respective pectin diet for two weeks. The control group was fed the control diet during the complete course of the experiment. (b) Control diet contained 20% cellulose, pectin diets contained 5 or 15% LMP or HMP.

Body weight, health status and food consumption were monitored every 2-3 days. Feces samples were freshly collected per cage at day 0, d3, d6, d9, d12, and d14. Serum as well as small and large intestine of the mice were collected after euthanasia at d14. The samples were immediately frozen and stored at -80°C until further analysis.

3.2.3 Peach peel extract preparation

Frozen peach peel was minced with a grinder to a fine powder and subsequently lyophilized. The prepared peach peel powder was further used for peach peel extract (PE) preparation as described in Steigerwald *et al.* 2023. Therefore, 1 g of lyophilized peach peel powder was mixed with 10 ml of ammonium bicarbonate buffer according to the method of Björkstén *et al.* with slight modifications (Björkstén et al. 1980). The extract was incubated for 2 h at 4°C under continuous shaking. Afterwards, $CaCl_2$ was added to a final concentration of 100 mM and incubated overnight at 4°C. In the following, the extract was centrifuged for 30 min at 12,000 x g and the supernatant was filtrated until 0.45 µm. Afterwards the extract was dialyzed against dH_2O and lyophilized. Finally, the lyophilized PE was reconstituted in a minimum volume of dH_2O and protein concentration was determined using Bradford protein assay (Bradford 1976). The extract was stored at -20°C until further use.

3.2.4 Peach allergy mouse model

Three different protocols for sensitization and provocation were compared to establish a strong and homogeneous peach allergy mouse model for the study. For all protocols, female CBA/J mice were used and the humane endpoint due to animal welfare reasons was defined by a drop of body temperature over 2°C, decline of general health according scoring system (Table 10) or body weight loss above 20%. In all examined schedules, core body temperature and symptoms of the mice were monitored up to 30 min after each oral exposure and challenge.

Table 10: Symptom score sheet.

Symptom Score	0	1	2
Behaviour	normal	calm	apathetic
Fur	normal	slightly ruffled	ruffled
Stool	normal	soft	with mucus
Body temp.	Δ < -1°C	Δ < -1 to -2°C	Δ > -2°C

In the first schedule, mice were sensitized 3 x via intraperitoneal (i.p.) injection of 200 µg PE or PBS (in 200 µl; n = 6) using aluminum hydroxide (alum) as adjuvant (1 mg per mouse) at d0, d14 and d28. This was followed from d35 on by oral provocation via intragastric (i.g.) administration of 500 µg PE or PBS (in 200 µl) every second day up to a maximum of 8 provocations (Figure 5).

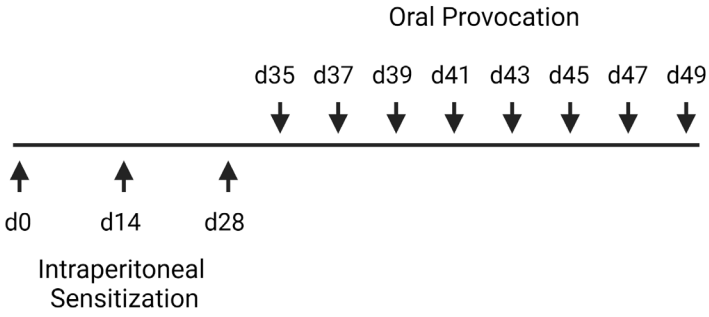

Figure 5: First sensitization protocol.
Mice were sensitized via i.p. injection of peach peel extract (PE) or PBS and alum, followed by up to 8 challenges via i.g. administration of PE or PBS.

In the second protocol, mice were sensitized 4 x via i.g. administration of 500 µg PE (in 200 µl; n = 5) or PBS (200 µl; n = 3) at d0, d7, d14 and d21. As adjuvants, either cholera toxin (CT) or staphylococcal enterotoxin B (SEB) were used (10 µg per mouse). Provocation was done by i.p. injection of 100 µg PE or PBS (in 200 µl) (Figure 6).

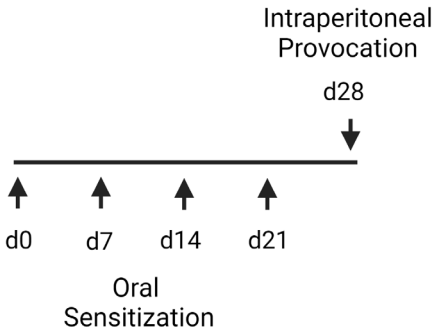

Figure 6: Second sensitization protocol.
Mice were sensitized by i.g. administration of PE or PBS with cholera toxin (CT) or staphylococcal enterotoxin B (SEB) as adjuvant. This was followed by one i.p. provocation with PE or PBS.

The third schedule was examined as described in the article of Steigerwald *et al.* 2023. Mice were sensitized via i.p. injection of 200 µg PE (in 200 µl; n = 5) or PBS (200 µl; n = 3) at d0, d7 and d12. Alum was used as adjuvant (1 mg per mouse). In the third week, the animals were exposed to 500 µg PE or PBS (in 200 µl) by i.g. administration every second day (d19, d21, d23) for a total of three times. The final provocation was done one week after the last i.g. exposure by i.p. injection of 100 µg PE or PBS (in 200 µl) (Figure 7).

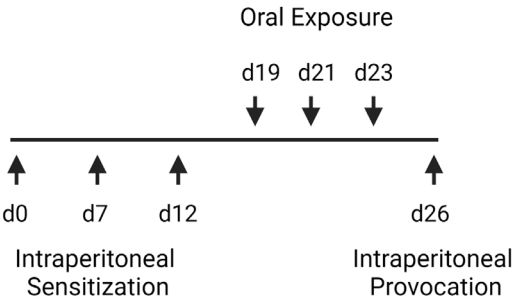

Figure 7: Third sensitization protocol.
Mice were sensitized by i.p. injection of PE or PBS using alum as adjuvant every week for a total of three weeks. This was followed by i.g. administration and i.p. provocation of PE or PBS.

3.2.5 Dietary pectin intervention in peach allergic mice

Dietary pectin intervention was performed in peach allergic mice. Female CBA/J mice were sensitized and challenged as described in section 3.2.4. Mice of the control groups (PBS or allergic control) were fed a control diet containing 20% cellulose starting two weeks before sensitization until the end of the experiment. Mice in the primary-prophylactic pectin intervention group were fed 20% cellulose control diet for one week, followed by 15% pectin diet starting 7 days before sensitization (d-7). In the secondary-prophylactic intervention group, mice were fed the 20% cellulose control diet until start of the 15% pectin diet after sensitization (d12) (Figure 8).

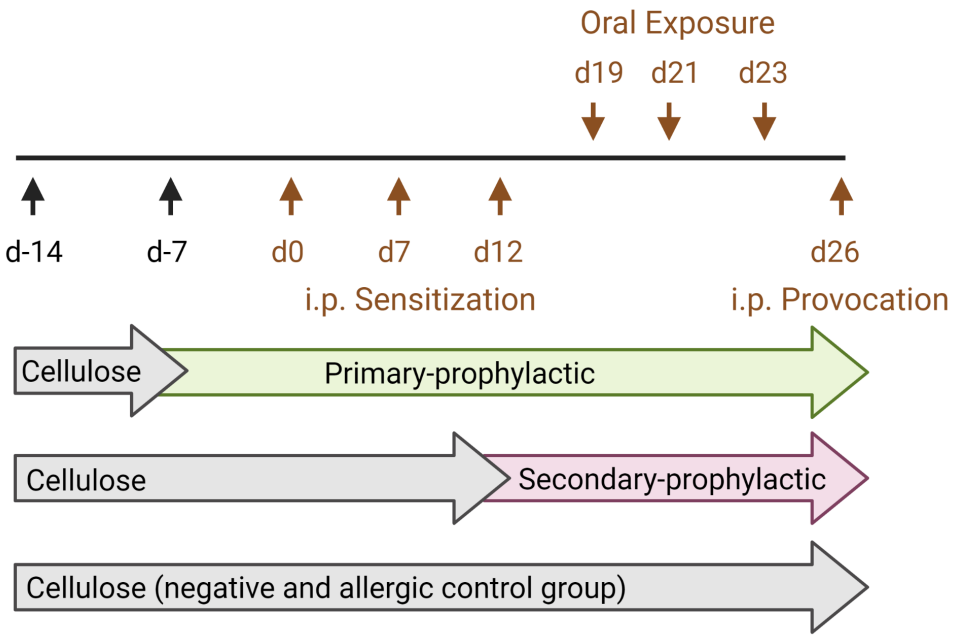

Figure 8: Pectin intervention schedule.
For pectin intervention, CBA/J mice were sensitized by i.p. injection using peach peel extract (PE) once per week for a total of three weeks. This was followed by oral exposure of PE three times and finally mice were challenged with i.p. injection of PE. A diet containing 15% HMP or LMP was use for dietary intervention. For primary-prophylactic pectin intervention, pectin feeding started 7 days before sensitization. Secondary-prophylactic intervention started after sensitization of the mice. The negative and allergic control groups were fed a 20% cellulose control diet.

3.2.6 Preparation of natural Pru p 3

Natural Pru p 3 (nPru p 3) was purified from freshly peeled peaches as described by Toda *et al.* 2011. For peach protein extraction the method described in section 3.2.3 was used. After filtration, the clarified extract was dialyzed three times overnight against 50 mM NaOAc, pH 5.3. n Pru p 3 was subsequently purified from the filtrate by two step chromatography. The extract was further applied to a cation exchange column using 50 mM NaOAc pH 5.3 as running buffer with 0 to 1 M NaCl gradient for elution (Toda et al. 2011). nPru p 3 containing fractions were further purified by size exclusion chromatography and PBS as running buffer. Fractions containing the pure protein were pooled and total protein content was determined using the BCA assay. Purity of nPru p 3 was assessed by Coomassie-stained SDS-PAGE and intact secondary structure by means of circular dichroism (Steigerwald et al. 2021).

3.2.7 Determination of protein concentration

Protein concentrations were determined using BCA Protein Assay Kit for purified proteins according to the manufacturer's recommendations. With this quantification method, Cu^{2+} is reduced to Cu^{+} by proteins in alkaline medium, followed by detection of Cu^{+} by bicinchoninic acid (BCA) and colorimetric protein quantification. For determination of protein concentrations in PE, Bradford assay was used. Here, proteins bind to Coomassie dye in an acidic environment resulting in a spectral shift from 465 nm (reddish brown) to maximum 610 nm (blue). This allows colorimetric protein quantification (Bradford 1976).

3.2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Proteins of PE and nPru p 3 were mixed with sample buffer (6% SDS, 40% glycerine, 0.1% bromphenoleblue, 60 mM Tris, pH 8.0) and incubated for 5 minutes at 95°C. Subsequently, proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10 µg/lane; length: 0.5 cm;

thickness: 1.5 mm) under reducing as well as non-reducing conditions using 16% acrylamide gel (Laemmli 1970) and visualized using GelCode blue Stain Reagent.

3.2.9 Immunoblot

To detect potential residual nsLTP in pectin samples, nPru p 3 and/or pectin samples were subjected to SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane by semi-dry blotting (Kyhse-Andersen 1984), followed by blocking with TBS blocking buffer. The membrane was subsequently incubated with a cross-reactive anti-Pru av 3 (cherry nsLTP) rabbit serum. Non-reactive rabbit pre-immune serum and secondary antibody were used as control. To detect bound IgG, horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and enhanced chemiluminescence (ECL) were used for visualization. The area under the curve (AUC) was analyzed using ImageJ version 1.53c and values were normalized to blank values. Blank values were set as 0, the positive control nPru p 3 was set as 1 (Steigerwald et al. 2021).

For inhibition experiments, nPru p 3 and/or PE were subjected to SDS-PAGE (50 ng/cm; lane length: 1.2 cm; thickness: 1.0 mm) under non-reducing conditions. Semi-dry blotting, blocking and antibody-incubation were performed as described previously. For dose-dependently inhibit antibody-binding, BSA or nPru p 3 were added to the rabbit serum in decreasing concentrations (1, 0.1 and 0.01 µg/ml). Non-reactive rabbit pre-immune serum was used as control. To detect bound IgG, HRP-labelled goat anti-rabbit IgG and ECL were used (Steigerwald et al. 2023).

To examine reactivity of mouse serum, nPru p 3 and/or PE were subjected to SDS-PAGE (10 µg/cm, lane length: 1.2 cm; thickness: 1.0 mm) under non-reducing conditions and transferred to a nitrocellulose membrane by semi-dry blotting as described previously. The membrane was incubated with serum pools of mice treated with PE or PBS (basal and final time points; 1:1000 diluted in working buffer) for 2 h at RT. Afterwards, the membrane was washed 3x with washing buffer for 5 min each and incubated with AP-labelled rat anti-mouse IgG1 for 1.5 h at RT. To detect bound IgG, NBT/BCIP solution was added until the protein bands were visible. The reaction was stopped by addition of dH_2O (Steigerwald et al. 2023).

3.2.10 Histological analysis

Histological analysis of the murine intestine was performed by Dr. Gonzalez-Menendez and Dr. Quintanilla-Martinez from the Institute of Pathology and Neuropathology, Comprehensive Cancer Center in the University Hospital Tübingen. Longitudinal sections of the jejunum of CBA/J mice were collected and fixed in 4% formalin and in the following embedded in paraffin. Sections of 5 µm thickness were prepared and stained with hematoxylin & eosin (H&E) for morphologic analysis.

3.2.11 Preparation of intestinal homogenates

Jejunum (10 cm length) was collected and Peyer's patches were removed. The tissue was washed with cold PBS, cut in small pieces and immediately frozen in liquid nitrogen. Subsequently, the frozen tissue was minced using mortar and pistil and the obtained powder was resuspended in 300 µl of cold PBS containing 1x protease inhibitor. Samples were centrifuged at 12,000 x g for 20 min and supernatant was transferred to fresh tubes. The protein concentration of the intestinal samples was determined using BCA assay. Following, protein concentrations was adjusted to 5 mg/ml using PBS and stored at -80°C for further analysis (Blanco-Pérez et al. 2019).

3.2.12 Measurement of allergen-specific and total antibody levels and mMCPT-1 levels in serum and intestinal homogenates

For determination of antigen-specific antibodies, 50 µl of nPru p 3 or PE were coated on microtiter plates (5 and 50 µg/ml, respectively; in coating buffer) overnight at 4°C. After blocking with 10% FCS in PBS for 2 h at RT, 50 µl of serum or intestinal homogenates were incubated 2 h at RT. Anti-mouse IgE antibody (1:1000) was incubated for 1 h at RT, followed by 30 min incubation of HRP-labeled streptavidin (1:2000). For detection of antigen-specific IgG1 (sIgG1) and sIgG2a, HRP-conjugated goat anti-mouse IgG1 (1:2000) or rabbit anti-mouse IgG2a (1:2000) were used. Antigen-specific antibodies were detected by addition of TMB-substrate

by measurement of the absorbance at 450 nm. Detection of total IgE, total IgG, total IgA as well as mMCPT-1 was performed using commercial ELISA kits according to the manufacturer's instruction (Table 7).

3.2.13 Measurement of T cell-derived cytokines

Spleens and mesenteric lymph nodes (MLNs) from CBA/J mice were collected and cells were isolated by manual disruption. Cells were seeded at 10^5 cells/well in a 96-well round bottom plate and were stimulated using 10 ng/ml Phorbol-12-myristat-13-acetat (PMA) and 1 µM Ionomycin for 72 h. Supernatant was collected and cytokine levels were determined using ELISA. Therefore, 50 µl of purified IL-5 capture antibody or IFNγ capture antibody (1:1000) were coated on microtiter plates in coating buffer overnight at 4°C. After blocking with 10% FCS in PBS for 2 h at RT, 50 µl of supernatant were added in duplicates (dilution 1:10 for IFNγ and undiluted for IL-5) and incubated for 2 h at RT. After washing for four times, plates were incubated with 50 µl of biotinylated anti-IFNγ or anti-IL-5 detection antibody (1:1000) for 1 h at RT. Subsequently, 50 µl of HRP-labeled streptavidin solution (1:2000) were added and incubated for 30 min at RT. Cytokines were detected by addition of 100 µl TMB-substrate by measurement of the absorbance at 450 nm. IL-4 and IL-13 were determined by ELISA with commercial reagent kits following the manufacturer's instruction.

3.2.14 FACS analysis of infiltrating immune cells in the lamina propria

Dissociation of the lamina propria (LP) was performed following a protocol from Weigmann *et al.* with slight adaptations (Weigmann et al. 2007). Small and large intestines of the mice were harvested, fat tissue and Peyer's patches were removed and the intestines were washed with cold PBS, opened longitudinally and cut in 1 cm pieces. The samples were further washed in 1x HBSS solution containing 5 mM DTT at 37°C for 20 min. Intestine pieces were subsequently passed through a 100 µm cell strainer and incubated in pre-digestion solution for 20 min at 37°C using slow rotation. The samples were again passed through a 100 µm cell strainer followed by repeated incubation in pre-digestion solution. Afterwards, the intestine

pieces were washed using 1x HBSS containing 10 mM HEPES, passed through a cell strainer and incubated in digestion solution for 20 min at 37°C using slow rotation. The samples were subsequently passed through a 40 µm cell strainer and the flow through was collected in cold FCS. The isolated cells were repeatedly washed in cold PBS, counted and used for further analysis by FACS.

Therefore, single cell suspensions of LP cells underwent Fc block with anti-CD16/32 followed by staining with extracellular antibodies, viability dye and if applicable nuclear staining (Table 6). Data were acquired using FACS Symphony and analyzed via FlowJo. Gating was performed as shown in Figure S2 and Figure S3.

3.2.15 Enzymatic pectin degradation

For enzymatic pectin treatment, a solution of Fructozym® (Table 4) was prepared (mixed 1:30 in H_2O (*v/v*)) and 5% or 10% (w/v) of the respective pectin was added and mixed at 40°C for 4 h under continuous stirring (Steigerwald et al. 2021). The enzymes were provided by Erbslöh Geisenheim GmbH, Geisenheim, Germany.

3.2.16 Protein and pectin precipitation

Protein precipitation from pectinase treated pectin was performed following the protocol from Niu *et al.*, with slight modifications (Niu et al. 2018). Ice-cold acetone was added to a 10% enzyme-treated pectin solution as described in section 3.2.15 (ratio 4:1 [v/v]) and incubated overnight at -20°C. Samples were centrifuged for 10 min at 15.000 x g, supernatants were discarded ant the protein pellets (upper white layer) were re-suspended in PBS without disturbing the pectin pellets (bottom brown layer) and immediately used in the posterior immunoblot.

Pectin precipitation was done using $CaCl_2$ as reported by Lević *et al.*, with slight modifications (Lević et al. 2007). Pectin was enzymatically treated as indicated in section 3.2.15. In the following, 10% enzyme-treated pectin was precipitated by addition of $CaCl_2$ to a final concentration of 100 mM and incubated overnight at 4°C under continuous shaking. The samples were centrifuged at 15.000 x g for 10 min,

and the supernatant containing the protein content was collected and subsequently used for immunoblot analysis as indicated in section 3.2.9.

3.2.17 Mediator release assay using humanized rat basophil leukemia (huRBL) cells

The mediator release assay was performed following the protocol established by (Vogel et al. 2006). huRBL-30/25 cells were harvested at the stationary phase, seeded at 1 x 10^5 cells/well in a 96-well plate and incubated overnight with human Pru p 3-sIgE-reactive serum at 37°C (specific IgE=21.5 kUA/L referring to CAP class 4). After washing, cells were stimulated with antigen for 1 h at 37°C (nPru p 3, nPru p 3 spiked in 5% pectin or 5% pectin alone). Initially, 1.5 µg/ml nPru p 3 or 0.05 µg/ml nPru p 3 were used, followed by serial dilutions. Total release values were revealed by lysing the cells with 1% Triton X-100. Afterwards, supernatant was collected and the specific mediator release triggered by cross-linking of receptor-bound IgE on the surface of the huRBL cells by allergen binding was measured at 405 nm and estimated as percent of total β-hexosaminidase release. The application of human serum was approved for use in the study by the local ethical committee of the Institut Universitari Dexeus of Barcelona (Spain) (Steigerwald et al. 2021).

3.2.18 16S rRNA analysis of the microbiome

Microbiome analysis of the pectin supplementation experiments was performed by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany (MRI). Dr. Oleg Krut and Dr. Csaba Miskey at the Paul-Ehrlich-Institut, Langen, Germany (PEI) performed the microbiome analysis of the pectin intervention experiments. Therefore, bacterial DNA was extracted using either the NucleoSpin DNA Stool Kit (MRI) or the QIAmp PowerFecal pro DNA Kit (PEI). DNA yield was quantified using either the Invitrogen Qubit dsDNA HS assay (MRI) or the QuantiFluor ONE dsDNA System Kit (PEI). Samples were adjusted to a final DNA concentration of 1 ng/µl and the sequencing library for 16S V3 (MRI) or V3 and V4 (PEI) rRNA amplicon high throughput sequencing was prepared and paired-end

sequencing was performed by Illumina MiSeq sequencing as described previously (Bender et al. 2023).

Data processing was performed using R studio (MRI) or Qiime2 (PEI) and demultiplexed fastq sequences were processed using DADA2 (v.1.22.0) (Callahan et al. 2016) Amplicon sequence variants (ASVs) were taxonomically assigned using the SILVA138 database (Quast et al. 2013).

3.2.19 Statistical analysis

The results of the pectin characterization as well as the mouse experiments are represented as means ± SEM, and the data were statistically evaluated by Mann-Whitney U test or ANOVA ($\alpha = 0.05$). The statistical software was Graph Pad Prism version 9.2.0. For statistical analysis of the sequencing data, the processed 16S V4 rRNA amplicon high throughput sequencing data was analyzed in RStudio using the packages phyloseq (v.1.348.0) and ggplot2 (v.3.3.6) (MRI) (Bender et al. 2023) or using Qiime2 software (PEI).

4 Results

4.1 Risk assessment of pectin

This thesis was part of the international collaborative study “DIFAMEM”, with the aim to examine the effect of dietary pectin intervention in peach allergic patients. The pectins used in this study were extracted from apple or citrus fruits and therefore potentially contain residues of allergens that were present in the source material. As both fruits express nsLTPs, such as Mal d 3 in apple or Cit l 3 in citrus fruit, a potential risk of IgE-cross-reactivity and induction of allergic reactions after consumption of the pectin by peach nsLTP allergic patients needed to be excluded. In this context, the two pectins were characterized in regard of the putative amount of residual nsLTPs to assess the associated risks for allergics and to ensure their safety. Results described in this chapter were previously published in Steigerwald *et al.,* 2021.

4.1.1 Allergen threshold estimation

Allergen thresholds leading to OAS or anaphylaxis were estimated for the used pectins to assess the possible risk of clinical reactions in nsLTP-sensitized patients after pectin ingestion. In the aforementioned dietary intervention study, peach-allergic patients should consume dosages of 10 g pectin in 100 ml smoothie (10% pectin solution; 100 mg/ml). Unpublished data of previous clinical trials from the collaboration partners at Instituto de Investigación Biomédica de Malaga (IBIMA) indicated that consumption of 300 µg nsLTP might elicit anaphylactic symptoms in nsLTP-allergic patients, whereas consumption of around 10 µg nsLTP might induce OAS (personal communication). Accordingly, the nsLTP amount in the pectin samples that might lead to severe reactions was estimated as 3 µg nsLTP per ml of a 10% pectin solution or 0.1 µg nsLTP per ml of a 10% pectin solution to induce OAS.

4.1.2 nsLTP detectability is hampered by pectin matrix

To confirm a potential risk for development of allergic symptoms for the patients, the above estimated nsLTP thresholds needed to be detected in the HMP and LMP preparations that were used in the human study. Therefore, the detectable range of protein concentrations in pectin was examined by spiking decreasing concentrations (10 µg/ml to 0.01 µg/ml) of nPru p 3 as marker nsLTP into a 10% solution of HMP or LMP followed by immunoblot analysis (Figure 9a,b).

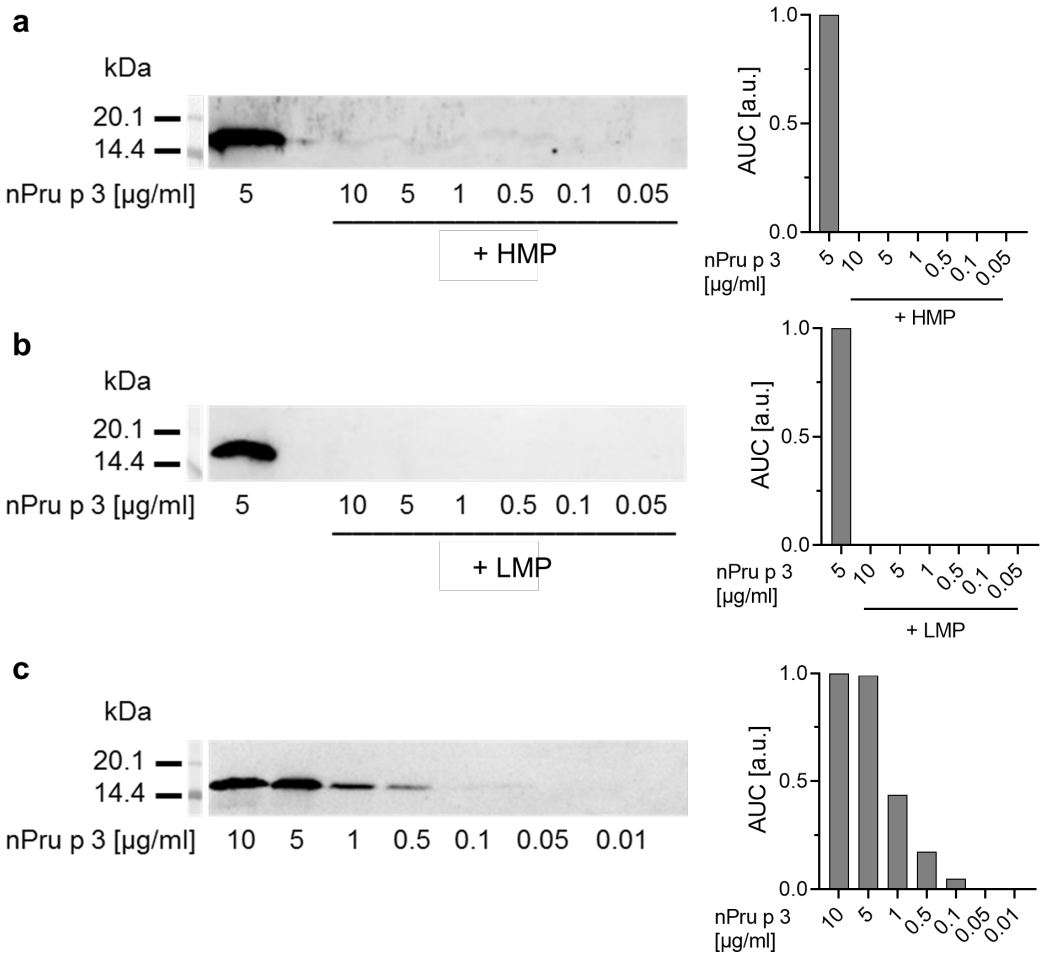

Figure 9: nsLTP detection in HMP or LMP by immunoblot.
Detectability of marker nsLTP nPru p 3 in (a) 10% high methoxyl pectin (HMP) or (b) 10% low methoxyl pectin (LMP) solution was evaluated via immunoblotting using a cross-reactive nsLTP rabbit antiserum. (c) Detection of nPru p 3 without pectin was used as control. The protein detectability was semi-quantified using the area under the curve (AUC) normalized to blank. Data published in Steigerwald *et al.* 2021.

The protein detectability in the matrices of the two pectins was compared to detection of 5 µg/ml nPru p 3 standard (without pectin) on the same blot as control as well as to detectability of decreasing nPru p 3 concentrations (Figure 9c). Detection intensity was semi-quantified as area under the curve (AUC) and normalized to the blank value. The results indicated, that protein concentrations down to 0.1 µg/ml nPru p 3 could be detected in the absence of pectin, but none of the nsLTP concentrations was detectable via immunoblot when spiked into either HMP or LMP.

As a second approach to identify nsLTPs in pectin, ELISA was used (Figure 10).

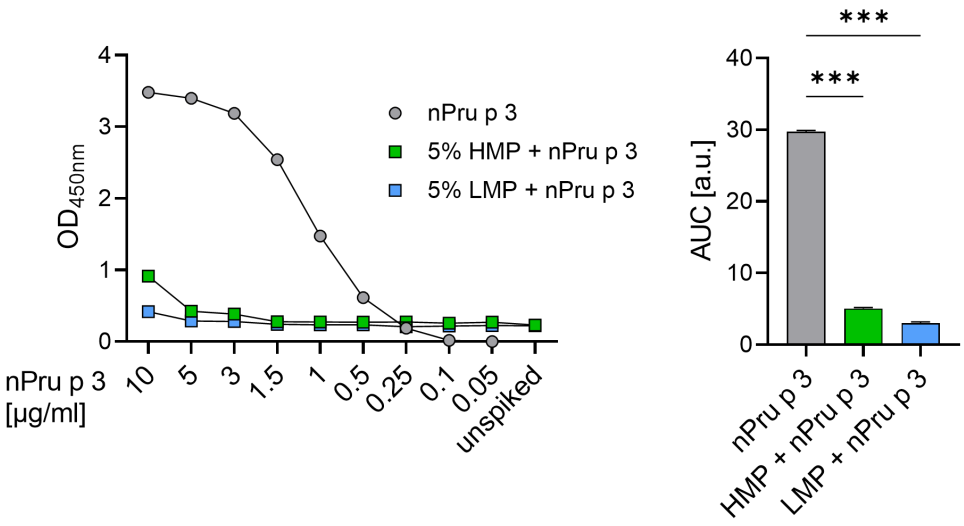

Figure 10: Detectability of nsLTPs in pectin matrix by ELISA.
Detectability of decreasing concentrations of the marker nsLTP nPru p 3 spiked in 5% HMP or LMP solution was examined by Pru p 3-specific IgE ELISA. The detectability was compared using the area under the curve (AUC). ***$p < 0.001$. Data published in Steigerwald *et al.* 2021.

Decreasing concentrations (10 µg/ml to 0.05 µg/ml) of nPru p 3 were spiked into 5% HMP or LMP solution and compared to nPru p 3 standard curve (10 – 0.05 µg/ml) without pectin as control. Twice the value of the buffer control was defined as limit of detection (LoD) for positive results (resulting in OD_{450nm} of 0.08). This method allowed nsLTP detection down to 0.25 µg/ml in the absence of pectin. The highest OD_{450nm} of around 3.5 was reached with 10 µg/ml of nPru p 3 without pectin. In comparison, when spiked into HMP the highest OD was measured around 1 and around 0.1 when spiked into LMP. In line with this, the detectability of nPru p 3 in

both pectins, reflected as AUC was significantly decreased, compared to nPru p 3 without pectin.

These results indicated that the protein detectability is strongly hampered by the pectin matrix of both HMP and LMP. Therefore, it was further necessary to increase the sensitivity of the detection methods.

4.1.3 Enzymatic treatment increases analytical sensitivity of nsLTP detection

To overcome the hampered protein detectability in the pectin matrix, samples of pectin and nPru p 3 were subjected to enzymatic digestion using the pectinase Fructozym® for 4 h at 40°C. Detection of 5 µg/ml nPru p 3 without pectin was used as control. IgG-reactivity of nPru p 3 was not affected by the enzymatic treatment as confirmed by SDS-PAGE and immunoblot (Figure 11). Comparison of nPru p 3 (P), heat-treated nPru p 3 at 40°C (hP) and pectinase-treated nPru p 3 (PF) revealed no effect of temperature or enzymatic treatment on the reactivity. The used pectinase Fructozym® (F) showed no IgG-reactivity.

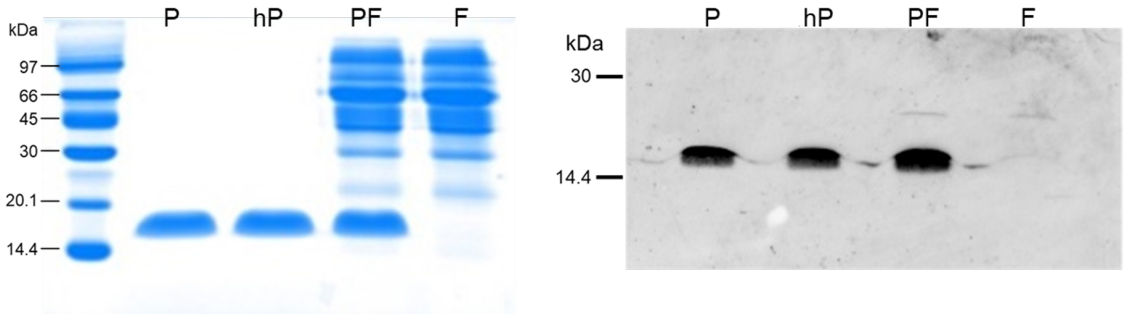

Figure 11: IgG-reactivity of nPru p 3 after enzymatic treatment.
Impact of pectinase treatment on IgG-reactivity of nPru p 3 was examined by SDS-PAGE and immunoblot. nPru p 3 was either heat-treated for 4 h at 40°C (hP) or incubated with the pectinase Fructozym® at 40°C for 4 h (PF) before analysis. Untreated nPru p 3 (P) or pectinase alone (F) was used as control. Data published in Steigerwald *et al.* 2021.

After enzymatic treatment, down to 5 µg/ml of the spiked nsLTP were detectable in both pectins, reflecting a recovery of about 40% in HMP and about 20% in LMP compared to the control referred to AUC (Figure 12). These results indicated that

the analytical sensitivity of the detection method could be increased by enzymatic pre-treatment using pectinase.

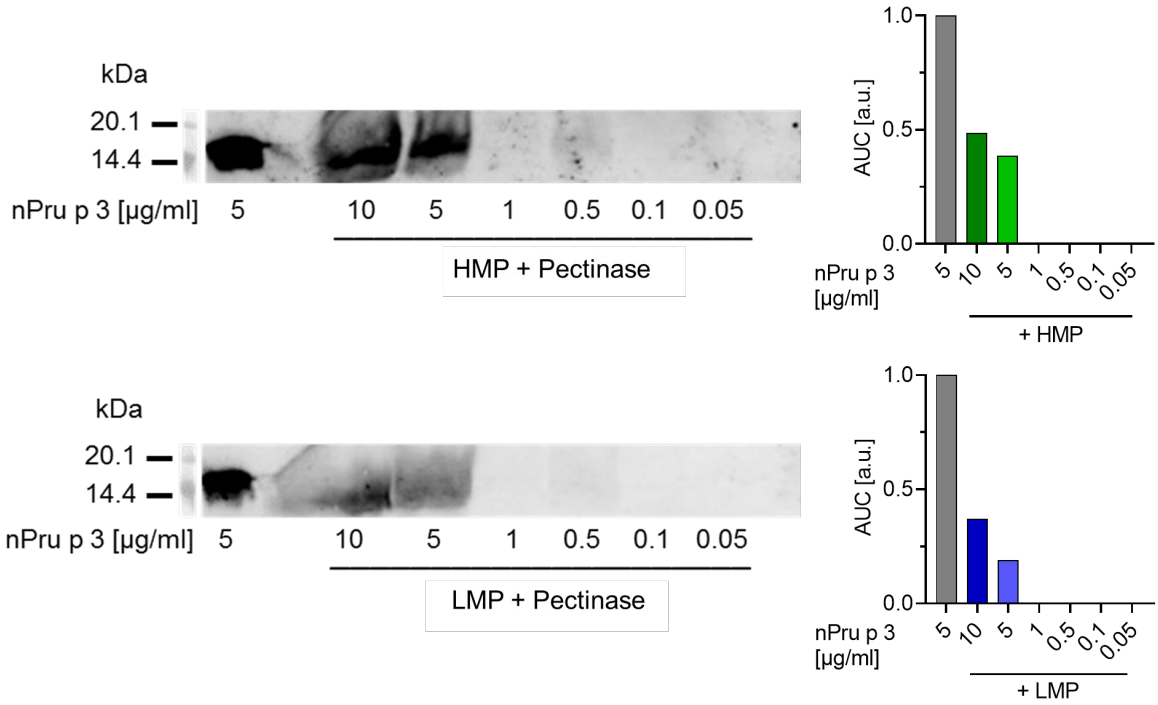

Figure 12: nsLTP detection in pectin upon enzymatic treatment.
Detectability of nPru p 3 in pectinase-treated 10% HMP or LMP solution was analyzed via immunoblot using a cross-reactive nsLTP rabbit antiserum. nPru p 3 (5 µg/ml) without pectin was used as control. Protein detectability was semi-quantified as area under the curve (AUC) normalized to blank. Data published in Steigerwald *et al.* 2021.

4.1.4 Enzymatic treatment allows detection of nsLTP thresholds in HMP

To evaluate a possible risk of pectin consumption for nsLTP-allergics, the method should be capable of detecting nsLTP thresholds eliciting OAS or anaphylaxis in the pectins. Therefore, nPru p 3 concentrations reflecting the estimated allergen concentrations leading to OAS (0.1 µg/ml) or anaphylactic reactions (3 µg/ml) were spiked into 10% pectinase-treated HMP or LMP and analyzed via immunoblot (Figure 13). Allergen without pectin served as positive control. Additionally, pectin samples without spiked nPru p 3 were analyzed regarding potential nsLTP content. The results were semi-quantified using AUC.

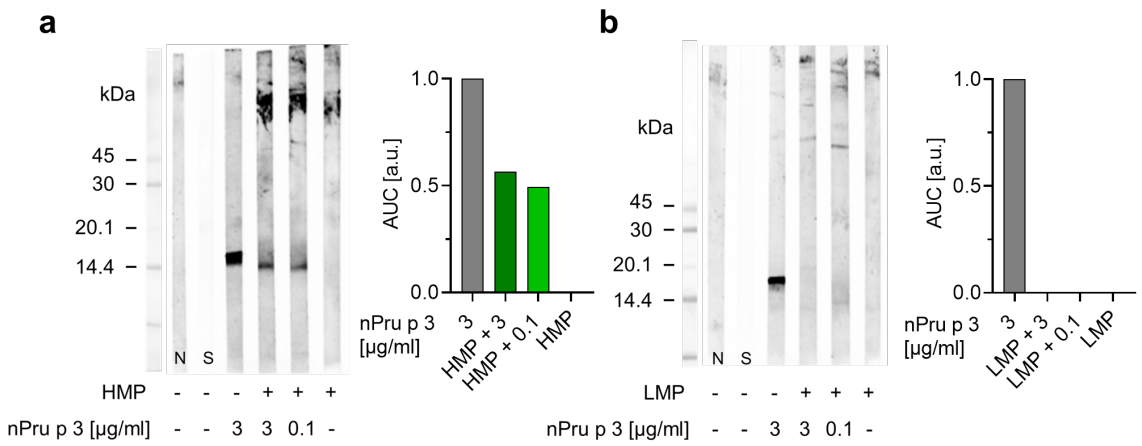

Figure 13: Detection of nsLTP thresholds in HMP and LMP.
Detection of nsLTP-spiked or unspiked samples of 10% pectinase-treated HMP (a) or LMP (b) via immunoblot. The spiked nsLTP concentrations represent the estimated nsLTP thresholds eliciting anaphylactic (3 µg/ml) or OAS (0.1 µg/ml). nPru p 3 without pectin was used as positive control. Detected bands were analyzed semi-quantitatively as AUC and normalized to blank. Incubation of the immunoblot with pre-immunization non-reactive serum was used as negative control (N) and incubation with buffer as secondary antibody control (S). Data published in Steigerwald *et al.* 2021.

Both spiked nPru p 3 concentrations reflecting the clinical thresholds were detectable in HMP (Figure 13a). Remarkably, no nsLTP was visible in the unspiked HMP sample. It could be concluded that the potential residual nsLTP content in HMP is below 0.1 µg/ml and therefore does not provide a relevant risk for peach allergics. Nevertheless, interference of the pectin matrix with protein detectability was still recognizable, as the AUC of 3 µg/ml spiked nPru p 3 decreased by almost 50% compared to the control. In contrast, when spiked in LMP, none of the two added allergen concentrations was visible, making a risk assessment for LMP consumption not feasible (Figure 13b).

4.1.5 Protein or pectin precipitation allows nsLTP detection in pectin

To further improve the sensitivity of nPru p 3 detection in the pectin matrix and to enable protein visualization in LMP, precipitation of either protein or pectin was performed. nPru p 3 alone, spiked in pectinase-treated pectins or pectin alone were treated with either acetone to precipitate the protein or $CaCl_2$ for pectin precipitation. Subsequently, samples containing the protein, were analyzed via immunoblot

(Figure 14). Semi-quantification of the detected protein bands was performed using the AUC.

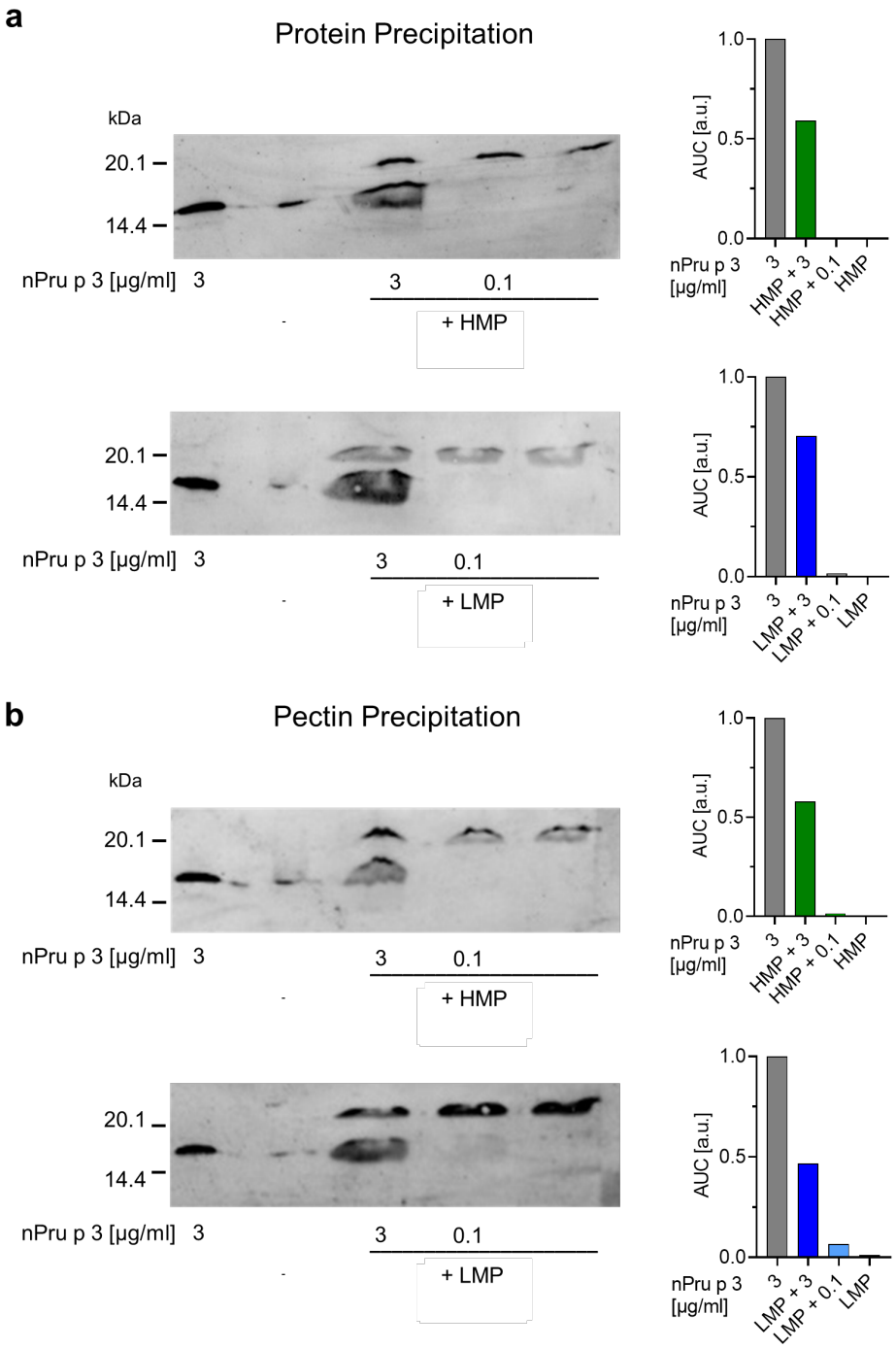

Figure 14: Protein or pectin precipitation increases nsLTP detectability.
Precipitation of protein (a) or pectin (b) and subsequent analysis via immunoblot. Spiked nPru p 3 concentrations representing the nsLTP threshold that elicit anaphylactic (3 µg/ml) or OAS (0.1 µg/ml) were spiked into 10% pectinase-treated HMP or LMP. Spiked pectins, unspiked pectins and nPru p 3 were used for precipitation. Detected bands were analyzed semi-quantitatively as AUC normalized to blank. Data published in Steigerwald *et al.* 2021.

The immunoblot results after protein precipitation indicated that the spiked nsLTP concentration representing the anaphylactic threshold of 3 µg/ml could be detected in both pectins (Figure 14a). The spiked nPru p 3 concentration reflecting OAS threshold was not visible in any of the two pectins as well as no protein band was detectable in the unspiked pectin samples. Similar results were obtained after pectin precipitation using $CaCl_2$ (Figure 14b). In agreement with the results above, the spiked concentration representing the anaphylactic threshold was detectable in both pectins as well as a weak band of 0.1 µg/ml nPru p 3 spiked in LMP, representing the OAS threshold. In both unspiked pectin samples no protein band was detectable, indicating no residual nsLTP content in the pectins.

4.1.6 Mediator release assay allows detection of low nsLTP concentrations in pectin

To further evaluate the sensitivity of protein detection in the pectin matrix, a functional cellular assay using huRBL cells was performed (Figure 15). Estimated nsLTP concentrations that might elicit anaphylactic reactions or OAS were spiked into 5% pectinase-treated HMP or LMP solution. Serum from a peach allergic patient with IgE to nsLTP was used for nsLTP-specific IgE binding and passive sensitization of the huRBL cells. Either nsLTP-spiked pectin samples, unspiked pectin or nPru p 3 were used to induce receptor cross-linking and mediator-release of the cells. The pectins used were enzymatically treated before analysis, to increase the detection sensitivity. The results revealed no substantial difference in the dose-dependent mediator-release of nPru p 3 spiked in pectin compared to the protein sample without pectin (Figure 15a,b). Furthermore, unspiked pectin samples did not cause mediator release at the concentrations tested in the dilution series. Additionally, mediator release induced by nPru p 3 compared to pectinase-treated nPru p 3 were examined, showing no effect of the enzymatic treatment on the assay (Figure 15c,d).

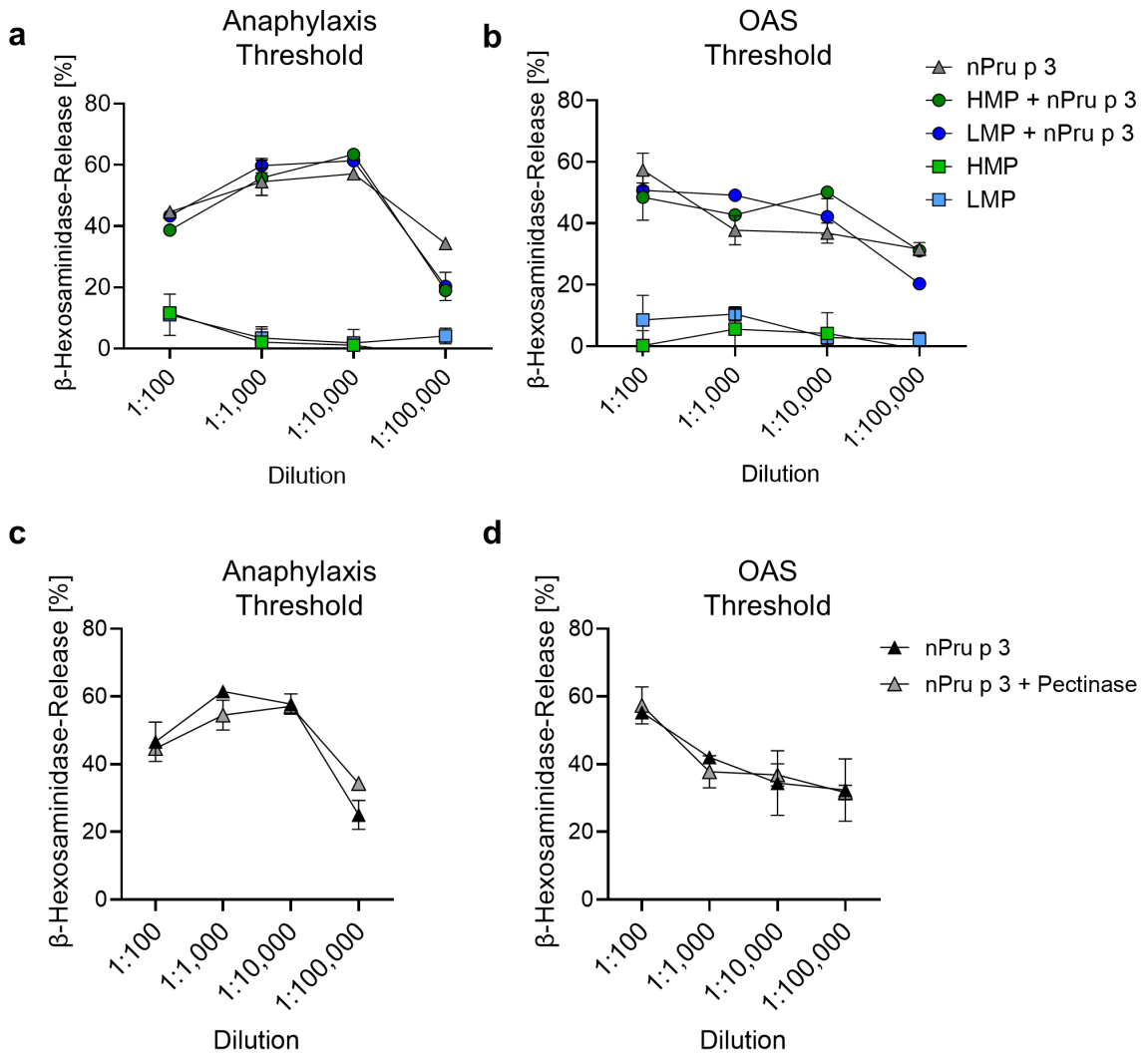

Figure 15: Detection of nsLTP thresholds by mediator release assay.
Mediator release assay using huRBL-30/25 cells passively sensitized with nsLTP-specific IgE. Stimulation was done using dilution series of pectinase-treated 5% HMP or 5% LMP, either unspiked, or previously spiked with nPru p 3 concentrations representing the anaphylactic (a) or OAS (b) threshold. The effect of pectinase-treatment on the detectability of nPru p 3 in this assay was examined for both used concentrations (c, d). Data published in Steigerwald *et al.* 2021.

Characterization of the two commercial pectins showed that different detection methods are necessary to evaluate the potential risk of nsLTP content in structurally different pectins. Nevertheless, the spiked nsLTP concentration representing the anaphylactic threshold was detectable in in both pectins by several assays, whereas no nsLTP could be observed in the unspiked pectin samples. These results indicated that the potential nsLTP content in both pectins very likely is below the threshold to elicit clinical reactions in nsLTP-allergic patients and consumption poses no risk for allergics.

4.2 Effect of pectin supplementation on naïve mice

Pectins are reported to promote beneficial health effects including promotion of gut homeostasis, modulation of microbiota composition and immune responses. These effects can either be mediated by direct interactions of pectin with immune cells and the epithelium or indirectly by microbial fermentation and production of mediators, such as SCFAs. Depending on the source material and extraction method, pectins can show a high structural variability, affecting also their solubility and fermentability by intestinal bacteria as well as the produced mediators (Blanco-Pérez et al. 2021). However, distinct effects, mediated by certain types of pectins are not yet identified with the consequence that the optimal type of pectin to achieve certain beneficial effects is unclear. To gain a deeper understanding of the potential mechanisms and immune-modulatory properties of structurally different pectin types, their effect on naïve and healthy mice was determined in this part of the study.

4.2.1 Impact of pectin supplementation on body weight and food intake

CBA/J, C57BL/6 and BALB/c mice were fed a control diet (20% cellulose) for two weeks, followed by different pectin diets (5% or 15% of HMP or LMP) for another two weeks (refer to Figure 4). The control group received the cellulose diet for the whole period of the experiment. To exclude any impact of the fiber intake on body weight of the mice by e.g. diarrhea, body weight and food consumption of the mice was monitored regularly (Figure 16).

The results showed that all mouse strains gained weight during the pectin supplementation period. However, differences in the total body weight gain were visible, as CBA/J mice fed a 15% HMP diet gained in average around 1 g body weight after 2 weeks, which is slightly less than the mice of the control group, that gained in average around 1.4 g (Figure 16a). In comparison, no differences in body weight gain were observed in C57BL/6 mice, as all diets led to a weight gain of approximately 1.1 g (Figure 16b). Interestingly, supplementation of 5% and 15% LMP significantly enhanced body weight gain in BALB/c mice to approximately 1.7 - 2 g, in comparison to 0.8 g in the control group, which correlates with a significant increase in food intake of these mice (Figure 16c).

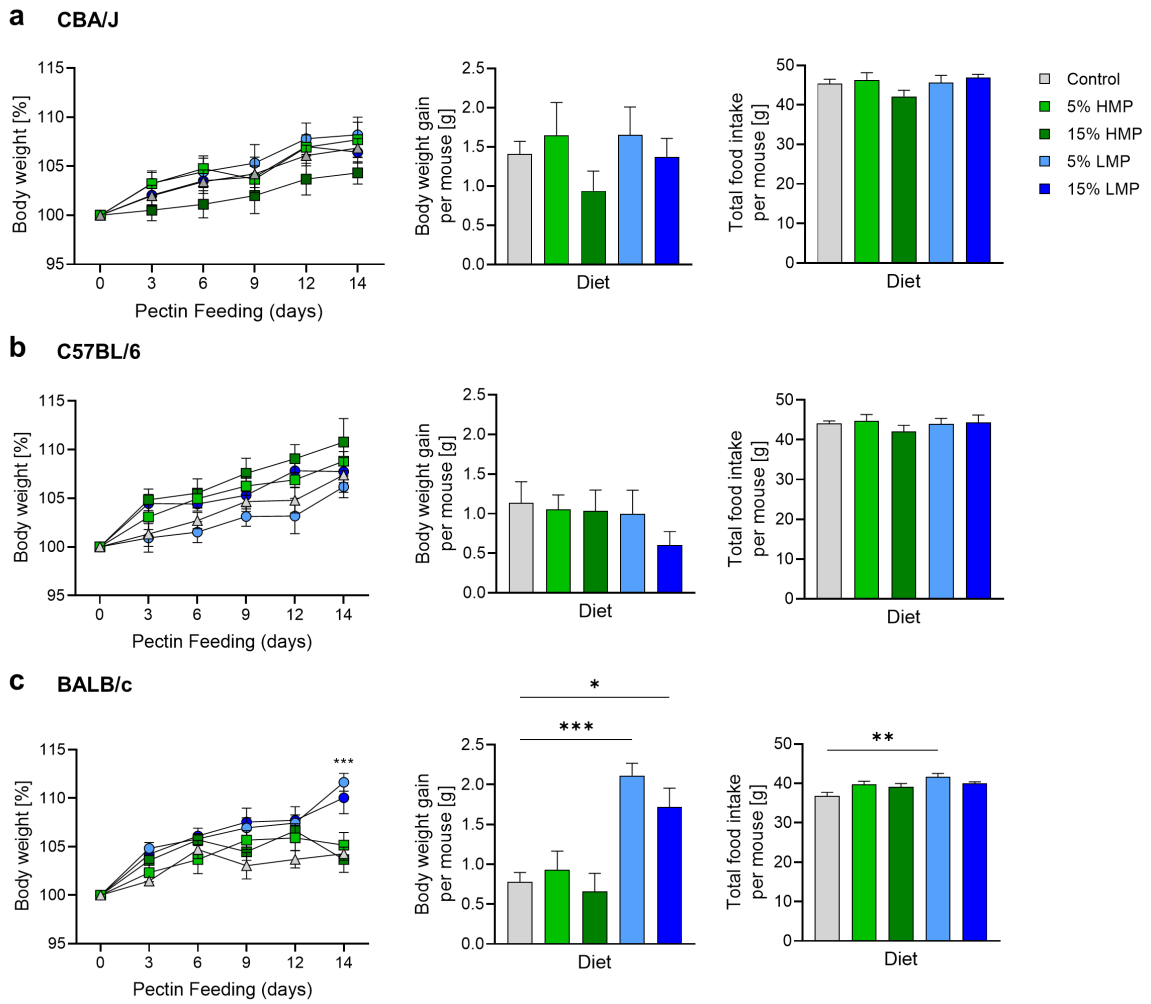

Figure 16: Body weight and food consumption during pectin supplementation.
Effect of pectin supplementation on body weight and food consumption in naïve (a) CBA/J, (b) C57BL/6 and (c) BALB/c mice was monitored regularly during the time of dietary pectin supplementation. n = 7-8; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

In summary, both pectins supplemented in both concentrations didn't induce a significant reduction of body weight gain in any of the mouse strains, whereas supplementation of 5% and 15% LMP enhanced the total body weight gain in BALB/c mice.

4.2.2 Consumption of HMP modulates microbiota composition in naïve mice

After evaluation of the effect of pectin supplementation on body weight and food intake, the possible effect on the gut microbiome of healthy mice was determined. Therefore, microbiome was analyzed in fecal samples of the three mouse strains during the pectin supplementation experiment at distinct time points. The microbiome analysis was performed by collaboration partners at the Max Rubner-Institut (Dr. Dominik Stoll and Dr. Melanie Huch, Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Karlsruhe, Germany), who kindly provided the results shown in this chapter and supported the analysis, interpretation and conclusion.

It could be observed in CBA/J mice that the microbial richness, calculated as Chao1 index was significantly decreased in the groups fed a HMP (5% and 15%) supplemented diet (Figure 17a). In comparison, the richness was not significantly affected with LMP diet (5% and 15%) or in the control group. Analysis of the mean relative abundance of ASVs in CBA/J mice showed that microbiota composition was substantially modified by HMP supplementation (Figure 17b). HMP feeding (5% and 15%) significantly increased ASVs assigned to *Bacteroides*, whereas supplementation of 5% HMP significantly increased the abundance of some members of the *Lachnospiraceae* family. In comparison, members of the *Muribaculaceae* family significantly decreased with HMP supplementation. Feeding of 15% HMP diet additionally decreased the abundance of several members of the *Lachnospiraceae* family and increased the abundance of *Escherichia/Shigella, Olsenella, Paludicola, Enterococcus* and *Faecalibaculum.*

Furthermore, the principal component analysis (PCoA) showed different clustering of the samples collected at day 0 (baseline value) compared to the samples collected at later time points of the study in CBA/J mice (Figure 18). In addition to the time-dependent clustering, certain clusters were observed depending on the supplemented pectin, as from day 3 on, samples of HMP diet clustered differently compared to the samples of LMP supplementation.

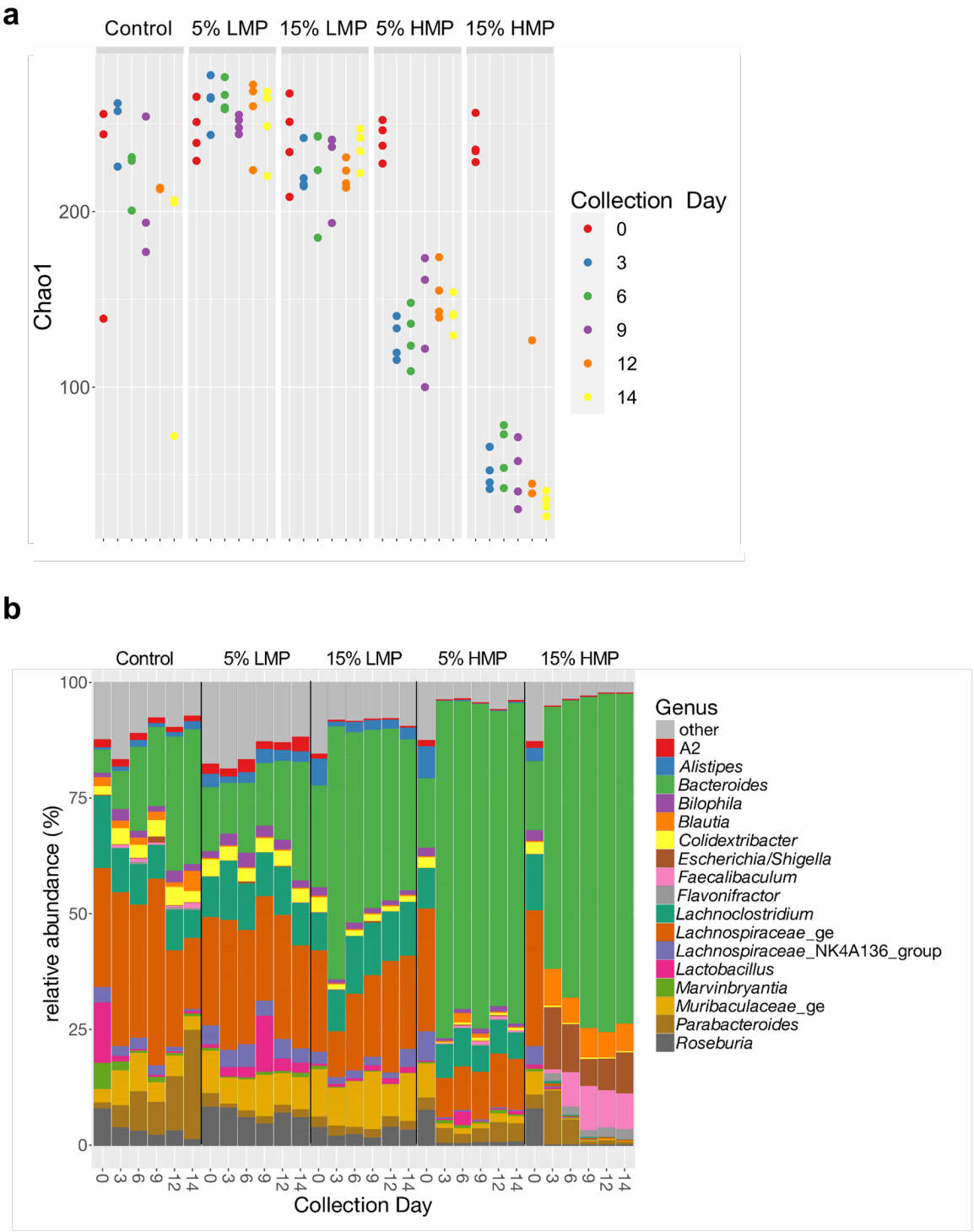

Figure 17: Effect of pectin on microbial richness and composition in CBA/J mice.
Microbial content was analyzed in feces samples of CBA/J mice at different time points. a) Richness was calculated using Chao1 estimator. b) Microbial composition was evaluated as the mean relative abundance of amplicon sequence variants (ASVs) that have been assigned to the 17 most abundant genera of the bacterial microbiota. The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

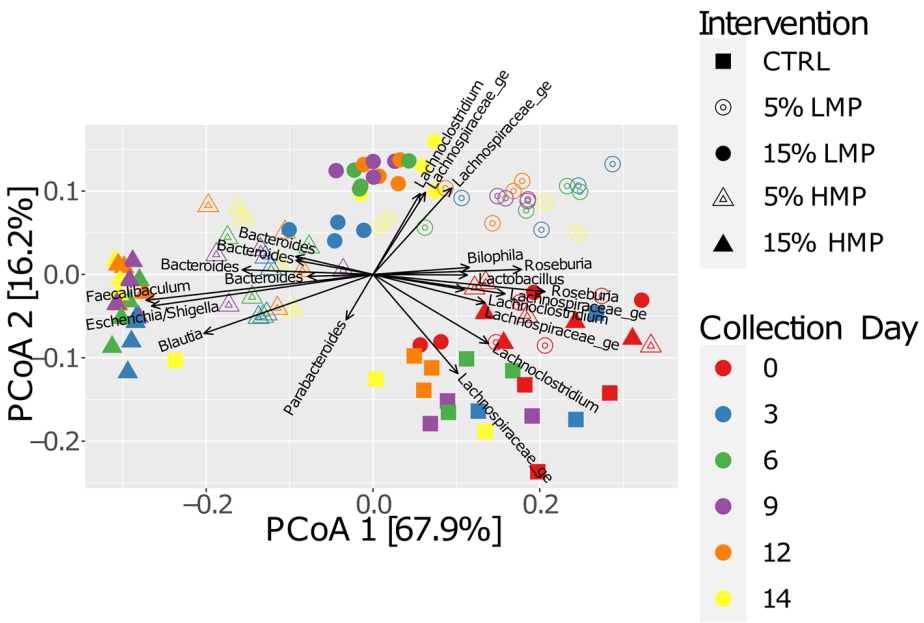

Figure 18: Beta diversity after supplementation of pectin to CBA/J mice.
Beta diversity of microbial content in the feces samples of CBA/J mice was calculated as Jensen-Shannon divergence and shown as Principal Component Analysis (PCoA). The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

Supplementation of different pectins also affected the microbial richness analyzed in fecal samples of C57BL/6 mice (Figure 19a). A significant reduction of the Chao1 index was observed from day 3 on, after supplementation of 15% HMP, whereas supplementation of both amounts of LMP did not significantly affect the richness in the fecal samples during the pectin supplementation period. However, supplementation of 20% cellulose also decreased the microbial diversity, as observed from day 6 on.

The relative abundance, analyzed as ASVs, showed a strong increase of ASVs assigned to *Bacteroides* especially in the group supplemented with 15% HMP (Figure 19b). However, also the diet containing 5% HMP or 15% LMP induced a slight increase of *Bacteroides* in the fecal samples.

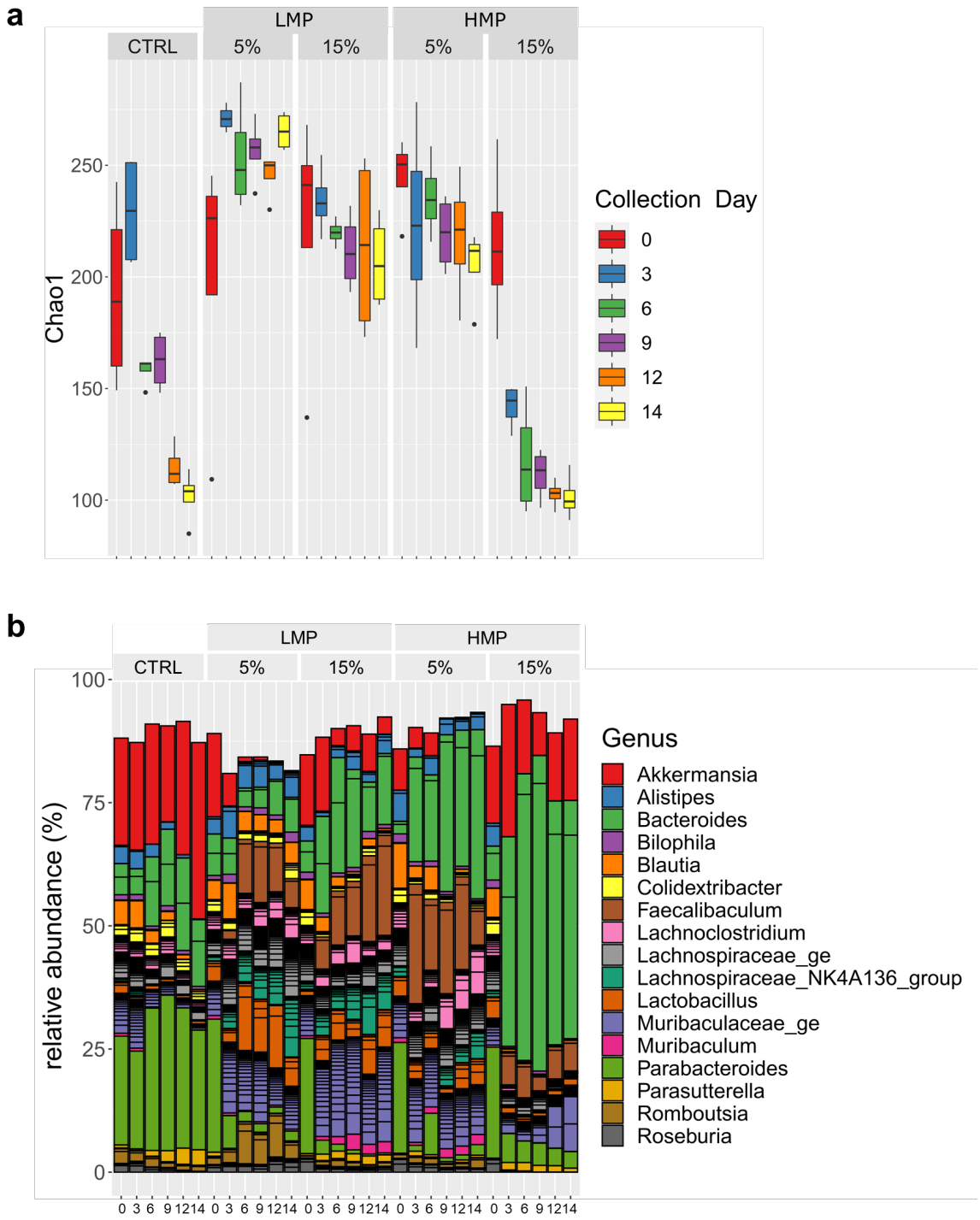

Figure 19: Effect of pectin on microbial richness and composition in C57BL/6 mice.
Microbial content was analyzed in feces samples of C57BL/6 mice at different time points. a) Richness was calculated using Chao1 estimator. b) Microbial composition was evaluated as the mean relative abundance of amplicon sequence variants (ASVs) that have been assigned to the 17 most abundant genera of the bacterial microbiota. The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

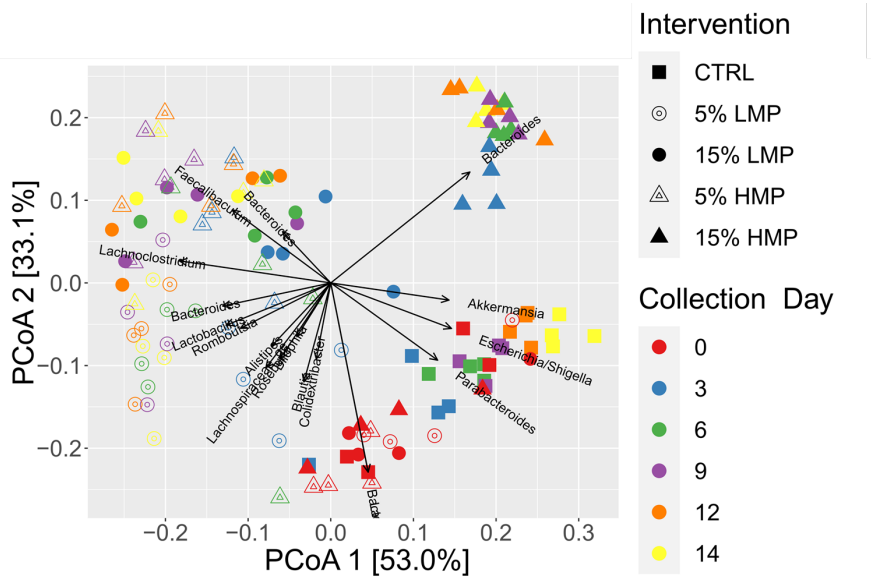

Figure 20: Beta diversity after pectin supplementation in C57BL/6 mice.
Beta diversity of microbial content in the feces samples of C57BL/6 mice was calculated as Jensen-Shannon divergence and shown as Principal Coordinates Analysis (PCoA). The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

The PCoA revealed clustering of the samples depending on the collection day as well as on the used pectin, as supplementation of either HMP or LMP led to distinct clusters as well as 5% or 15% supplementation. (Figure 20). This could be observed starting from day 3 of the feeding period.

Additionally, microbiota composition was analyzed in fecal samples of BALB/c mice during the pectin supplementation period. Comparable to the results observed in CBA/J and C57BL/6 mice, the microbial richness was significantly decreased after 3 days when 15% HMP was supplemented to the diet, which could also be observed in the control group from day 9 on (Figure 21a). In line with that, relative abundance of *Bacteroides* was strongly enhanced after HMP supplementation, especially with 15% HMP. This effect could also be observed in the cellulose group in this mouse strain (Figure 21b).

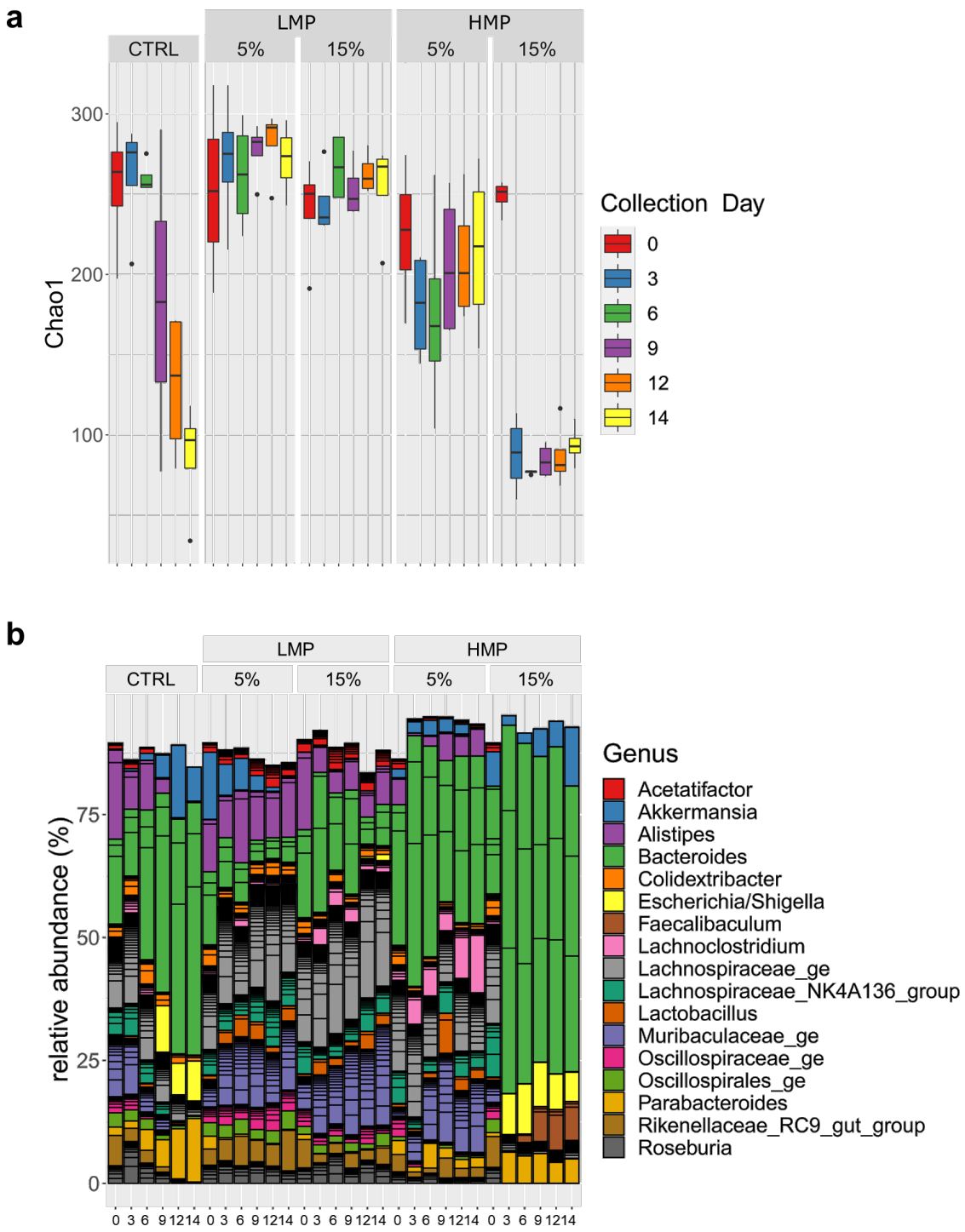

Figure 21: Pectin supplementation affects microbial richness and composition in BALB/c mice.
Microbial content was analyzed in feces samples of BALB/c mice at different time points. a) Richness was calculated using Chao1 estimator. b) Microbial composition was evaluated as the mean relative abundance of amplicon sequence variants (ASVs) that have been assigned to the 17 most abundant genera of the bacterial microbiota. The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

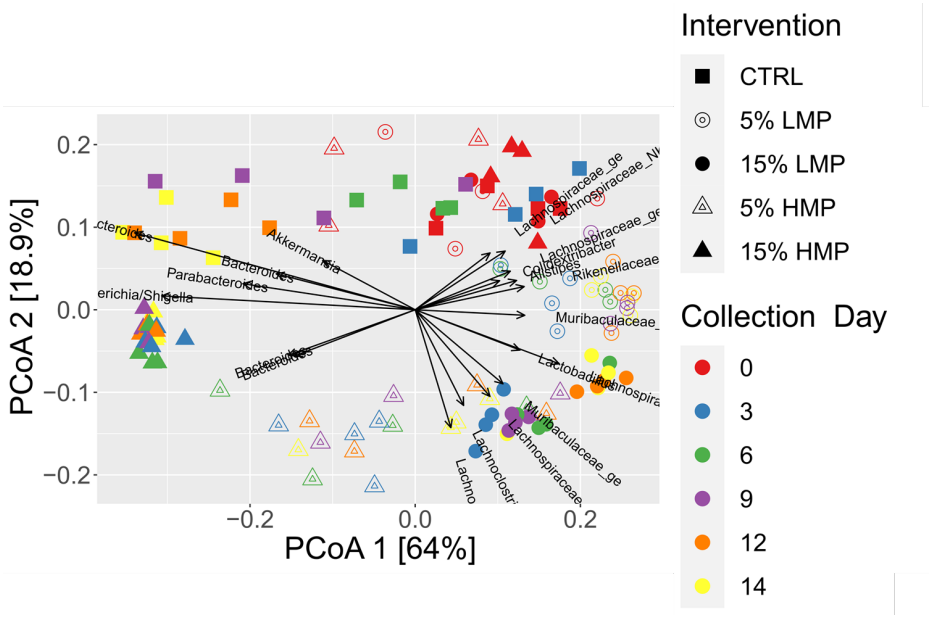

Figure 22: Beta diversity in BALB/c mice after pectin supplementation.
Beta diversity of microbial content in the feces samples of BALB/c mice was calculated as Jensen-Shannon divergence and shown as Principal Coordinates Analysis (PCoA). The data were generated and provided by Dr. Dominik Stoll and Dr. Melanie Huch from the Max Rubner-Institut, Karlsruhe, Germany.

Comparable to the results achieved in CBA/J and C57BL/6 mice, the PCoA of BALB/c mice showed a clustering of the samples collected at day 0 compared to the samples collected at later time points (Figure 22). Additionally, a distinct clustering could be observed between supplementation of HMP and LMP, as well as the used amount of pectin.

The analysis of microbial richness and diversity in the feces of the three mouse strains during pectin supplementation period revealed that 15% HMP significantly reduced microbial richness in all mouse strains after three days. In contrast, diet of LMP (5% or 15%) did not affect the microbial richness in the mice during the experiment. Furthermore, dietary supplementation of HMP, especially 15%, induced a significant increase of *Bacteroides*, that could be observed in all three mouse strains after 3 days of HMP supplementation.

4.2.3 Dietary supplementation with HMP modulates humoral immune response

In the following, possible immune-modulatory effects of structurally different pectins in naïve mice of three different strains (CBA/J, C57BL/6 and BALB/c) were investigated. Therefore, levels of total IgA, IgG and IgE were measured in the serum by ELISA (Figure 23).

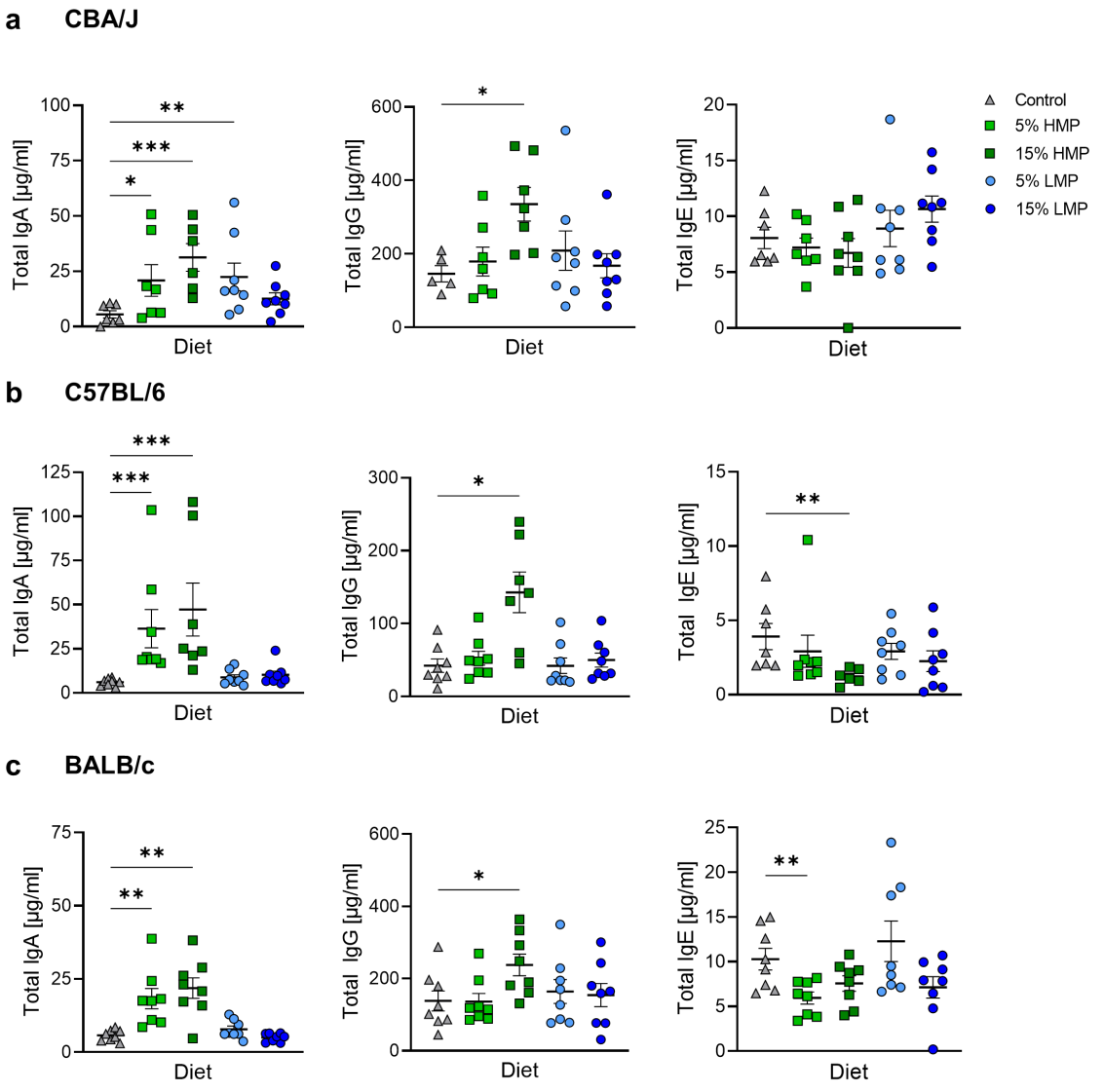

Figure 23: Modulation of humoral immune-response by pectin.
Total immunoglobulin (Ig) levels of IgA, IgG and IgE were measured after the pectin supplementation period in the serum of (a) CBA/J, (b) C57BL/6 and (c) BALB/c mice by ELISA. n = 7-8; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Significant and dose-dependent effects, especially after supplementation of HMP were observed. In all mouse strains, HMP (5% and 15%) supplementation induced a significant increase of total IgA. In CBA/J mice this increase of IgA could also be significantly determined with 5% LMP supplementation. Additionally, supplementation of 15% HMP induced a significant increase of total IgG levels in all mouse strains and decreased levels of total IgE in C57BL/6 and BALB/c mice when compared to the control. LMP (5% and 15%) induced no effect on the levels of IgG and IgE in any of the mouse strains.

The ratio of total IgG to total IgE revealed a significant increase induced by supplementation of 15% HMP in all three examined mouse strains, indicating a shift towards IgG production (Figure 24). In contrast, supplementation of 5% HMP, 5% LMP and 15% LMP did not affect this ratio in none of the mouse strains. Thus, HMP but barely LMP supplementation modulated the humoral immune response in all three analyzed mouse strains, characterized by enhanced total IgA production and increase of total IgG/IgE ratio.

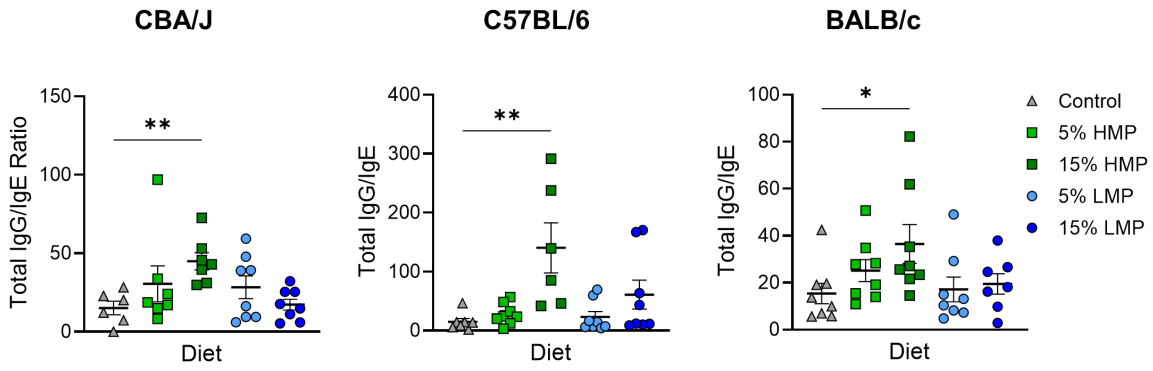

Figure 24: Total IgG/IgE ratio after pectin supplementation.
The ratio of measured total IgG levels to total IgE levels in the serum were examined in the three different mouse strains CBA/J, C57BL/6 and BALB/c. n = 7-8; *$p < 0.05$; **$p < 0.01$

4.2.4 Effect of different pectin types on intestine physiology and infiltrating immune cells

To investigate potential effects of pectin supplementation on intestine physiology, at the end of the two weeks supplementation, the length of three intestinal sections (small intestine, large intestine and caecum) was measured (Figure 25).

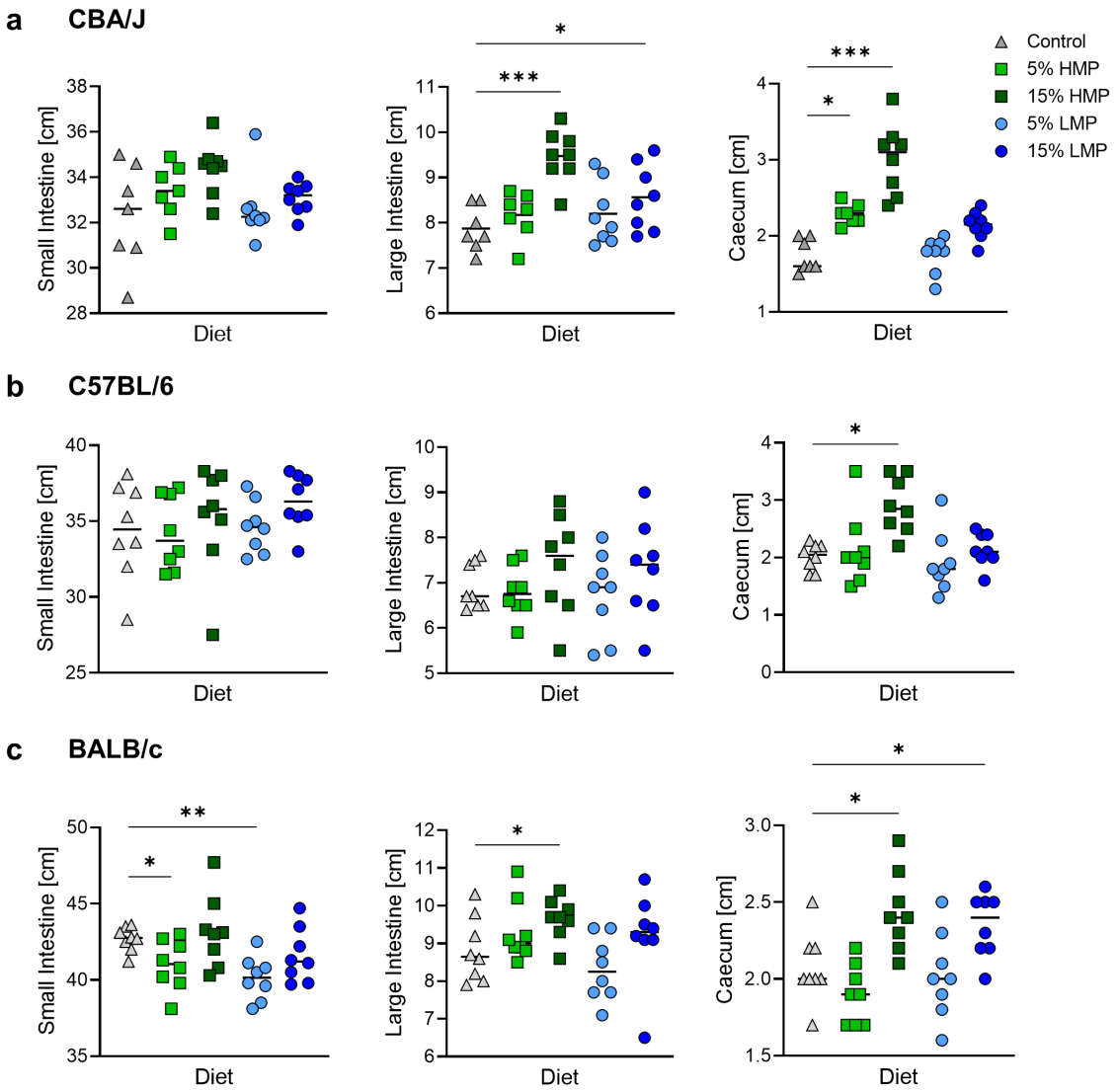

Figure 25: Intestine length after pectin supplementation.
The length of small intestine, large intestine and caecum was determined after two weeks of pectin or control diet in (a) CBA/J, (b) C57BL/6 and (c) BALB/c mice. n = 7-8; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Only feeding of 15% HMP led to slight enlargement of the small intestine in CBA/J and BALB/c mice when compared to the control mice. In contrast, in BALB/c mice, supplementation of 5% HMP and 5% LMP significantly reduced the small intestine length. Large intestine was significantly enlarged in CBA/J mice after 15% HMP and 15% LMP supplementation and in BALB/c mice with 15% HMP diet. This trend was observed in C57BL/6 mice as well, but failed to reach significance. Furthermore, 15% HMP diet correlated with a significantly enlarged caecum in all three mouse strains, observed as well with 5% HMP supplementation in CBA/J and 15% LMP in

BALB/c mice. No effect on caecum length could be observed with the other used pectin diets.

To exclude any adverse effects of the pectin-enriched diet, histological analysis of the jejunum were performed. H&E staining showed no inflammatory response after high doses (15%) of pectin supplementation on the intestine physiology and only scattered inflammatory cells were detected (Figure 26). The histological staining as well as the interpretation was performed by Dr. Irene Gonzalez-Menendez and Dr. Leticia Quintanilla-Martinez from the Eberhard-Karls University, Tübingen, Germany.

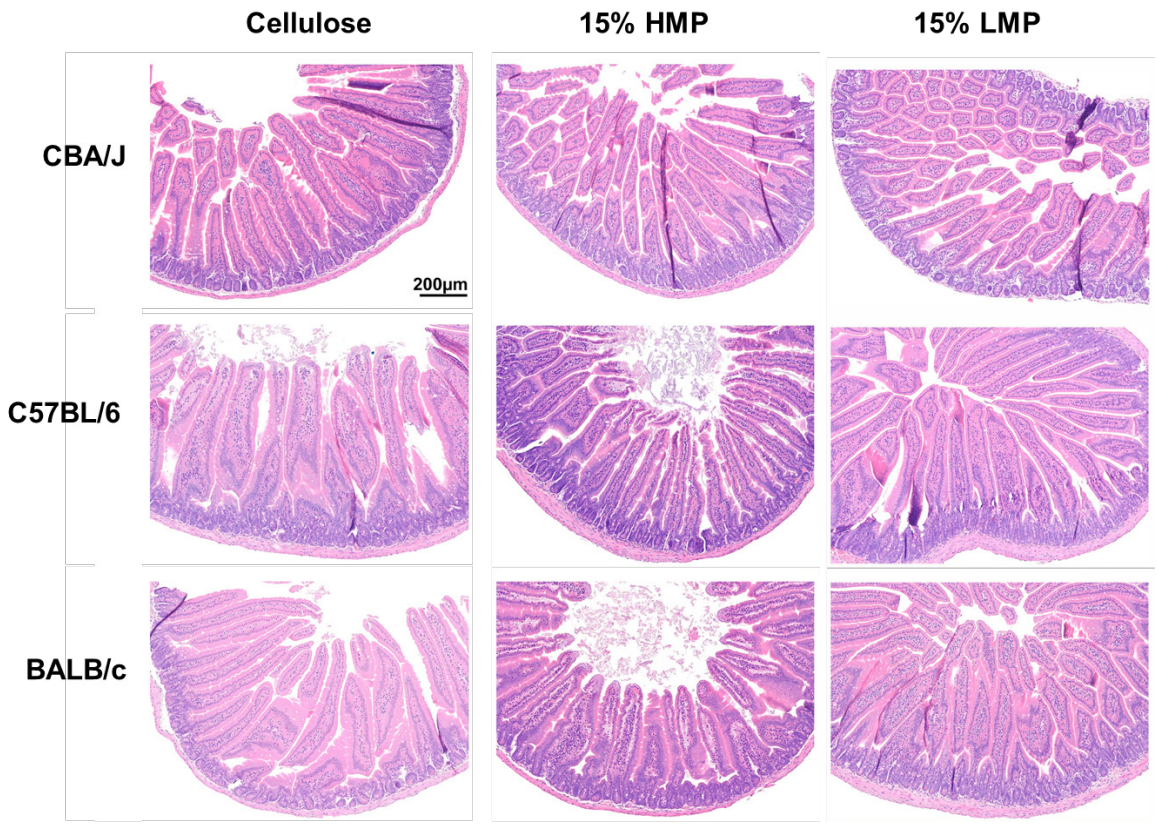

Figure 26: Histological analysis of jejunum after pectin supplementation.
Jejunum was histologically analyzed via H&E staining after the pectin supplementation. The effect of 15% HMP, 15% LMP or 20% cellulose supplemented diet on intestinal physiology is shown for the three mouse strains. Staining and analysis of the results was done by Dr. Gonzalez-Menendez and Dr. Leticia Quintanilla-Martinez (Eberhard Karls University,Tübingen, Germany) who provided the data shown in this figure.

Characterization of local immune-responses in the small intestine revealed that dietary supplementation of pectin, especially 15% HMP promoted changes in the balance of the infiltrating cells in the LP of CBA/J mice (Figure 27). Results showed that supplementation of 15% LMP led to decreased numbers of T cells. In comparison, supplementation of 15% HMP promoted enhanced levels of Th cells and induced decreased amounts of Tregs, cDCs, macrophages, eosinophils and mast cells. Dietary supplementation of 5% HMP induced enhanced levels of B cells, whereas 5% LMP could not alter frequencies of infiltrating cells.

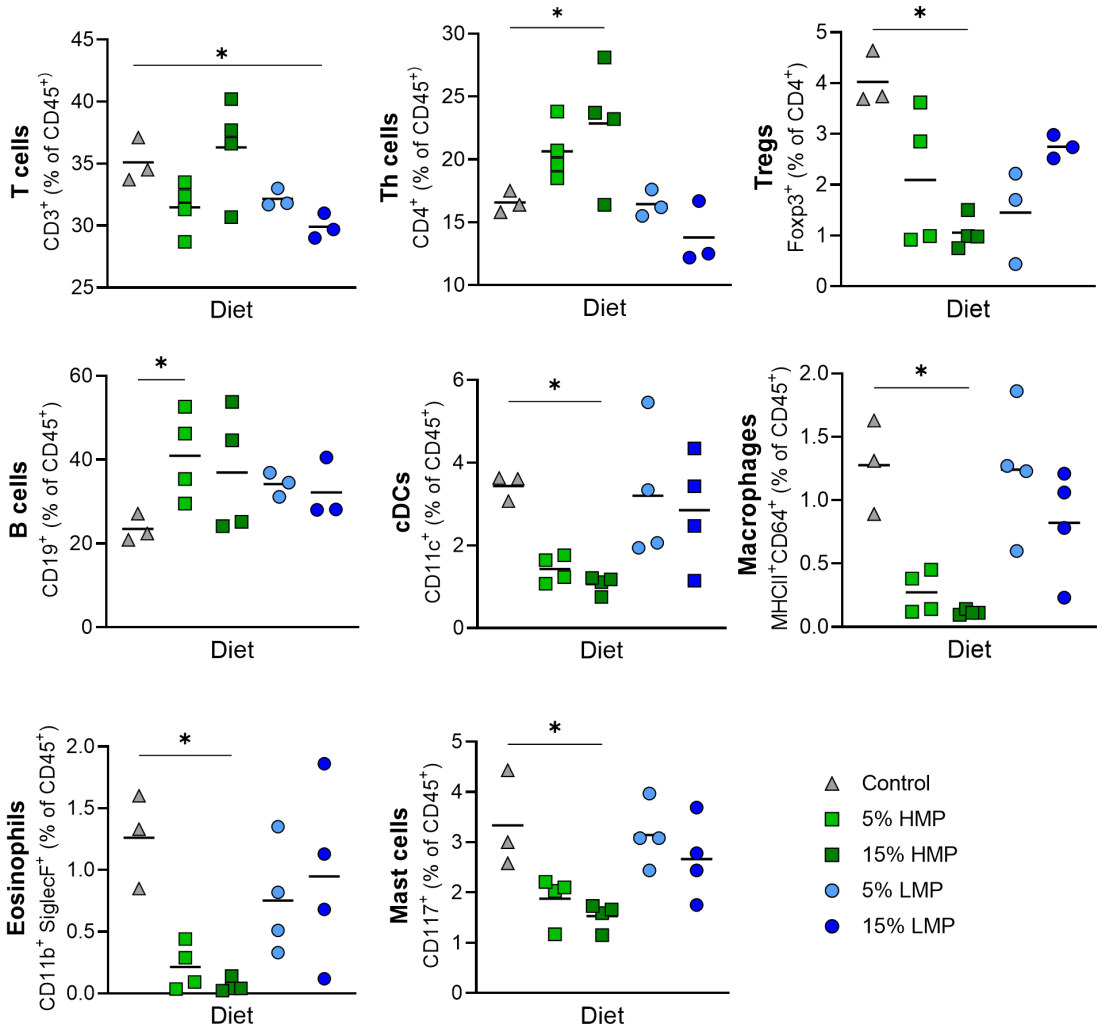

Figure 27: Impact of pectin supplementation on infiltrating cells in the lamina propria. Amount of different infiltrating cells in the lamina propria (LP) of the small intestine were analyzed by FACS, including T cells, T helper cells (Th cells), regulatory T cells (Tregs), B cells, conventional dendritic cells (cDCs) macrophages, eosinophils and mast cells. n = 3-4; *$p < 0.05$

The data showed that structurally different pectins exert different immune-modulatory effects. Especially supplementation of 15% HMP modulated levels of infiltrating immune cells in the LP, characterized by enhanced Th cells but reduced Tregs, cDCs, macrophages, eosinophils and mast cells. In contrast, dietary supplementation of LMP (15%) induced reduced numbers of Tregs but did not affect the abundance of other immune cells.

4.3 Establishment of a peach allergy mouse model

The results shown in section 4.2 indicated that dietary supplementation of pectin modulates the gut microbiota composition and affects immunological responses in healthy mice, depending on the amount and type of pectin used. However, these effects were observed in healthy mice and might therefore not reflect the modulatory capacity of pectin in case of disease. It was reported that pectin might positively affect the manifestation and severity of allergies by modulation of the microbiome (Trompette et al. 2014), whereas the effect on FA has not been studied yet. Therefore, this project aimed to investigate the immune-modulatory impact of a dietary pectin intervention in FA, particularly peach allergy. To examine the effect of pectin on FA, a mouse model is an effective tool to study the modulatory impact as well as the underlying mechanism. Thus, a model of experimental peach allergy was established in mice as a model of FA in this study. Due to previously obtained successful sensitization with nsLTPs, CBA/J mice were used for the allergy model. Results described in this chapter were partly published in Steigerwald *et al.*, 2023.

4.3.1 Characterization of peach peel extract

For the establishment of a peach allergy mouse model, PE (preparations see chapter 3.2.3) was used to sensitize and challenge the mice. Thus, prior to using the PE in the mouse model, it was characterized regarding its protein pattern and antibody reactivity (Figure 28).

A broad range of proteins could be detected in the PE, including the major peach allergen Pru p 3. The content of Pru p 3 was estimated as the major part of the total amount of extracted proteins. To confirm the presence of Pru p 3 in the extract, immunoblot and competition assay using nPru p 3 or BSA (negative control) as inhibitors, were performed. Here, nPru p 3-specific inhibition could be observed, identifying predominant 10 kDa band as nPru p 3 in the extract. In the following, PE was prepared following the standardized protocol described in section 3.2.3 and was used for the establishment of the peach allergy mouse model.

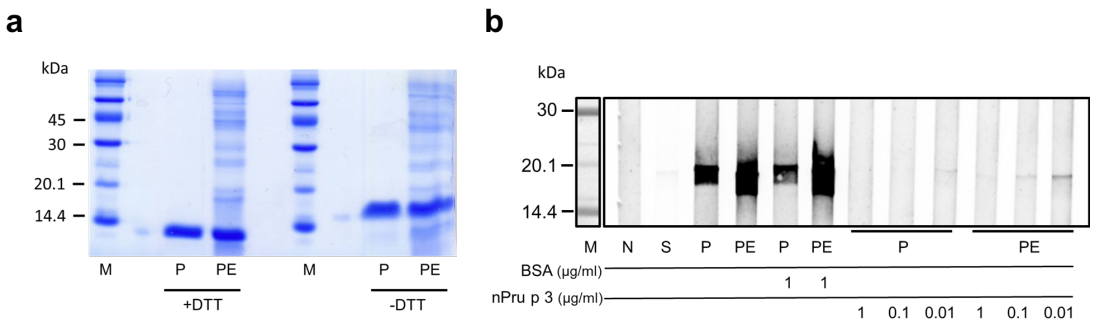

Figure 28: Characterization of peach peel extract.
Peach peel extract (PE) was characterized regarding protein pattern and reactivity. (a) Protein pattern was determined via SDS-PAGE and Coomassie staining under reducing (+DTT) or non-reducing (-DTT) conditions, natural Pru p 3 (nPru p 3; P) was used as reference. (b) nPru p 3 or PE were subjected to SDS-PAGE followed by immunoblot analysis. Reactivity was determined using nsLTP-reactive rabbit serum and inhibition of antibody binding by addition of increasing dosages of nPru p 3 or BSA as control and referred to molecular weight standard (M). Non-reactive serum (N) and secondary antibody alone (S) were used as controls. Data published in Steigerwald *et al.* 2023.

4.3.2 Intraperitoneal sensitization induces sIgE production but weak clinical signs

In order to establish a peach allergy mouse model, a homogeneous Th2-biased reaction of the mice was required. Therefore, different experimental schedules were evaluated and compared regarding induction of clinical sings as well as Pru p 3-sIgE in the serum. In a first attempt, mice were sensitized three times by i.p.-injection of PE and alum as adjuvant once per week (refer to Figure 5). This was followed by oral provocation with PE every second day up to a maximum of eight provocations. Body temperature of the mice was measured before and after each oral provocation and the mean drop of body temperature of the mice is shown for each time point (Figure 29a). In total, only two mice developed a drop of body temperature and reached the humane endpoint, defined by body temperature drop over 2°C. One mouse reacted after the second, the other after the sixth oral provocation. Levels of Pru p 3-sIgE in the serum were significantly increased after sensitization (Post-Sens) and after provocation (Final) in all of the PE-treated mice compared to the PBS controls (Figure 29b).

Nevertheless, to study the effects of pectin supplementation in a peach allergy mouse model, a strong and homogeneous clinical reaction of the mice was required.

Therefore, further strategies for the development of peach allergy in mice had to be examined.

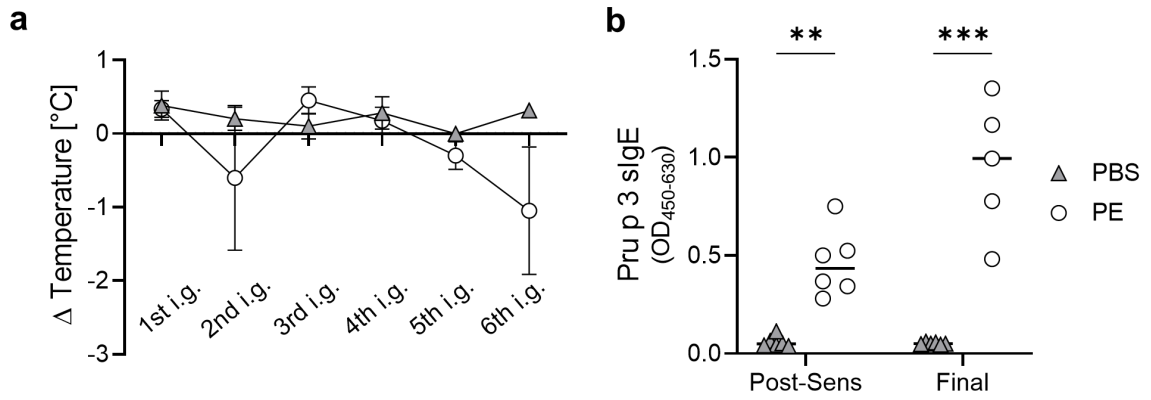

Figure 29: Allergy-related clinical signs after i.p. sensitization and oral provocation.
Drop of body temperature was measured 15 min before and every 15 min after each oral provocation and Pru p 3-specific IgE levels have been measured in the serum. (a) Mean body temperature of the mice 15 min after each provocation (1st – 6th) and (b) Pru p 3-specific IgE in the serum of the mice after sensitization (Post-Sens) or after provocation (Final). n = 6; $^{**}p < 0.01$; $^{***}p < 0.001$.

4.3.3 Oral sensitization induced clinical signs but low sIgE-response

Due to the weak clinical signs observed in the previous protocol using i.p. sensitization and oral provocation, a different scheme was applied. Here, PE was administered 4x orally to sensitize the mice, followed by one i.p. provocation with PE (refer to Figure 6). Additionally, usage of two different adjuvants (CT or SEB) during sensitization was assessed. Body temperature of the mice was measured 15 min before and every 15 min after provocation (Figure 30a). The results indicated a significant drop of body temperature 30 min and 45 min after provocation in both PE-immunized groups. However, measurement of the Pru p 3-sIgE in the serum revealed that after provocation only three mice out of five of the PE + CT group developed increased antibody levels compared to the baseline levels, whereas none of the mice immunized with PE + SEB developed Pru p 3-sIgE (Figure 30b).

The data showed, that oral administration of PE successfully induced homogeneous clinical reaction of the mice according to the allergy-related drop of body temperature of around 1.5°C, but was not capable to induce a homogeneous sIgE-response.

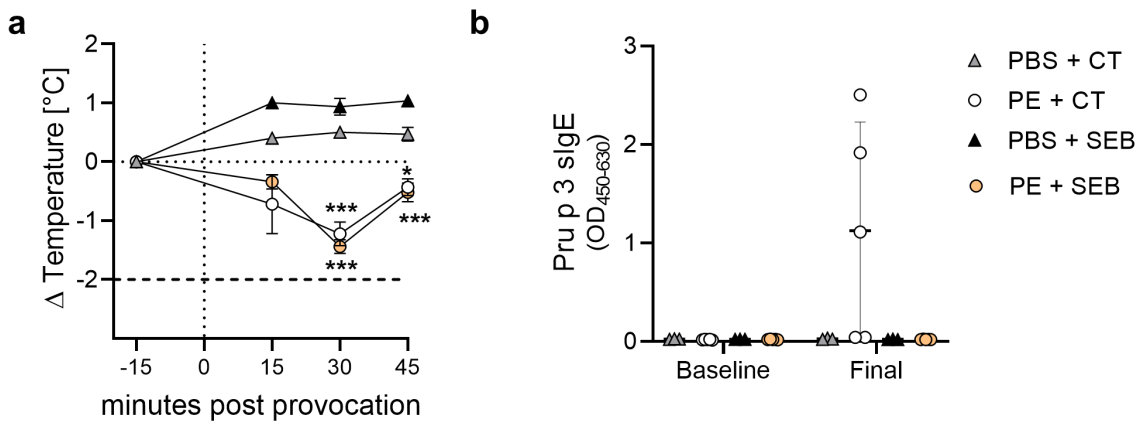

Figure 30: Allergy-related clinical signs after oral sensitization and i.p. provocation.
Mice were sensitized by intragastric (i.g.) application of PE or PBS as control, using cholera toxin (CT) or staphylococcal enterotoxin B (SEB) as adjuvants. (a) Body temperature of the mice was measured 15 min before and up to 45 min after provocation. (b) Pru p 3-specific IgE was determined in the serum of the mice before sensitization (Baseline) or after provocation (Final) by ELISA. n = 3-5; ***$p < 0.001$.

4.3.4 Induction of peach allergy by i.p. sensitization and i.p. provocation

Finally, the knowledge and experience from the previous experimental attempts to establish a peach allergy mouse model were used and the advantages of the prior tested schedules were taken into consideration: development of sIgE antibodies was successfully provoked by i.p. sensitization, whereas i.p. provocation led to a strong drop of body temperature. To facilitate an involvement of the intestinal tract in the FA model, PE was i.g. administered after sensitization, in the absence of adjuvants (refer to Figure 7). As described in section 3.2.4, body temperature of the mice was measured 15 min before and up to 45 min after the provocation. The results showed a significant decrease of the body temperature of around 1.2°C in average, of the PE-immunized mice after 15 min, and even stronger, by 1.9°C 30 min after provocation (Figure 31a). Furthermore, all mice immunized with PE showed significantly enhanced Pru p 3-sIgE levels after provocation (Final), whereas no effect of PBS-immunization was observed on the sIgE levels (Figure 31b).

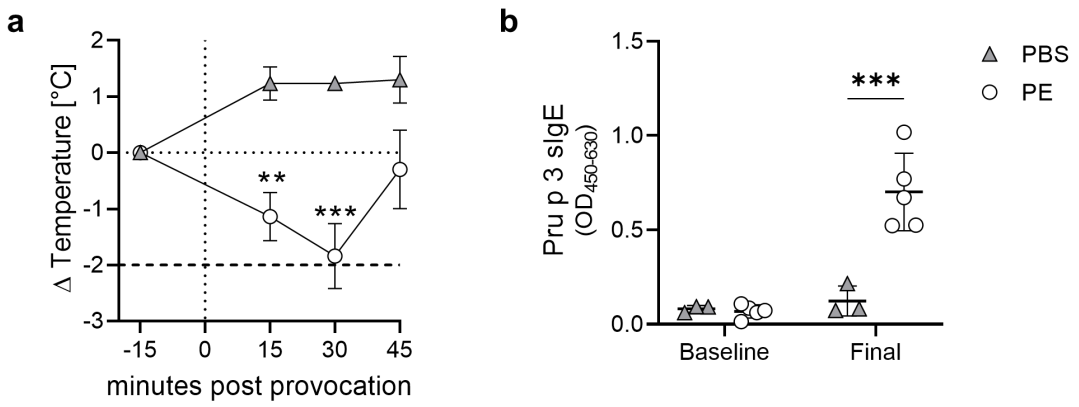

Figure 31: Body temperature and immune response after i.p. sensitization and i.p. provocation.
(a) Body temperature of the mice was measured 15 min before and up to 45 min after provocation (b) and Pru p 3-specific IgE was analyzed in the serum of the mice before sensitization (Baseline) or after challenge (Final). n = 3-5; $^{**}p < 0.01$; $^{***}p < 0.001$.

These results indicated that combination of i.p. sensitization and i.p. provocation together with oral administration of PE upon sensitization induced a homogeneous drop of body temperature as well as a significant increase of sIgE.

4.3.5 Characterization of the established peach allergy mouse model

The established peach allergy model in CBA/J mice was further characterized and confirmed. The following data are shown as combined data of two experiments, performed under the same experimental setting. Body temperature after provocation was significantly decreased by over 2°C in the PE-immunized group 15 min after provocation (Figure 32a, b). Analysis of the allergy-related symptoms via symptom score evaluation showed an increased score after each oral exposure (approximately 0.8 after 2nd and 1.2 after 3rd oral exposure) and the highest values of around 3 were observed after provocation (Figure 32c). The score included alteration of the behaviour, external observation of the fur and the stool as well as the drop of the body temperature (Table 10). The development of clinical signs is shown in Figure 32d, as the mouse treated with PE showed a calm behaviour and ruffled fur after challenge in comparison to the mouse of the control group, treated with PBS.

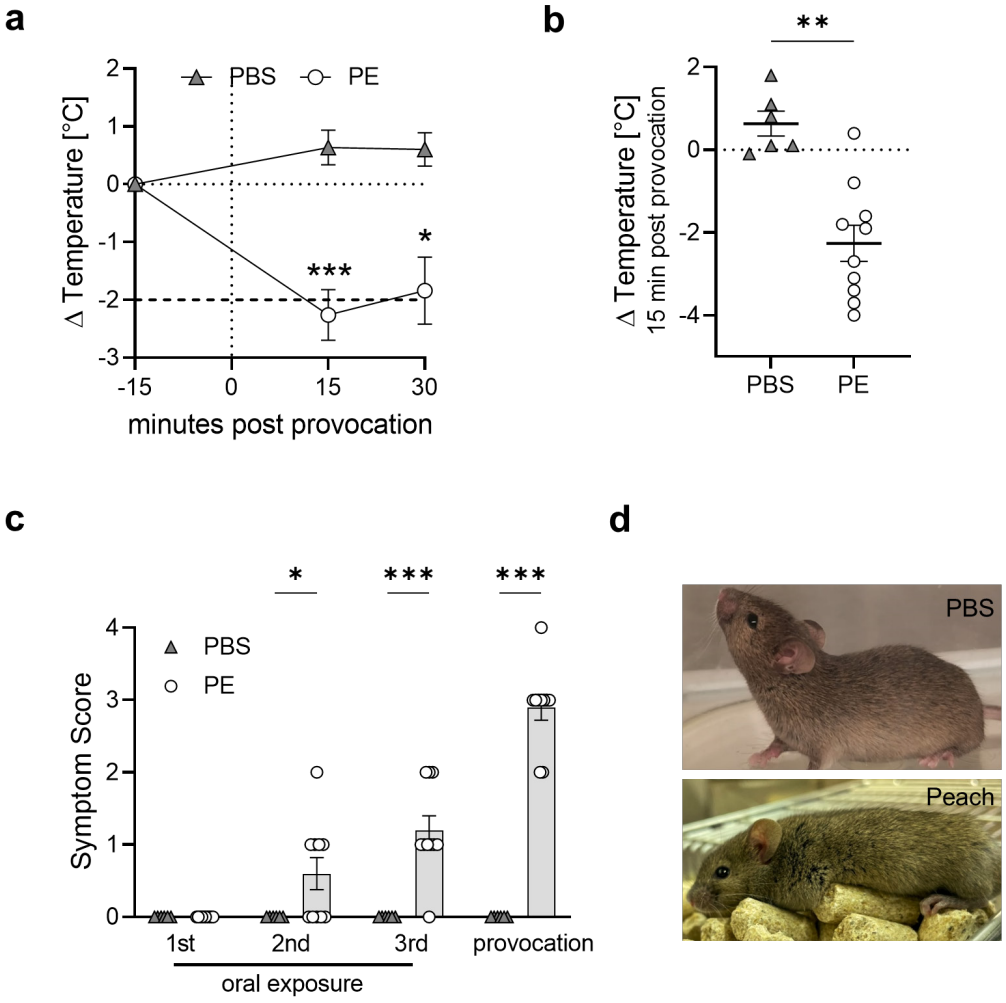

Figure 32: Confirmation of temperature drop and development of clinical signs.
Induction of clinical signs in the experimental model of food allergy. (a) Body temperature was measured before (-15 min) and up to 30 min after provocation. (b) Body temperature of individual mice 15 min after provocation. (c) Symptom score after each oral exposure and provocation. (d) PBS- or PE-treated mouse after provocation. n = 6-10, data presented as combination of 2 experiments performed under identical settings; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data published in Steigerwald *et al.* 2023.

The humoral immune response of the mice was examined by measurement of PE- and Pru p 3-specific antibodies in the serum. Mice immunized with PE developed a significant increase in PE-specific IgE, sIgG1 and sIgG2a (Figure 33a-c). Comparable results were observed for Pru p 3-specific antibodies (Figure 33d-f). The ratio of PE- as well as Pru p 3-specific IgG1 to sIgG2a was significantly increased in the PE group after provocation (Figure 33g-h).

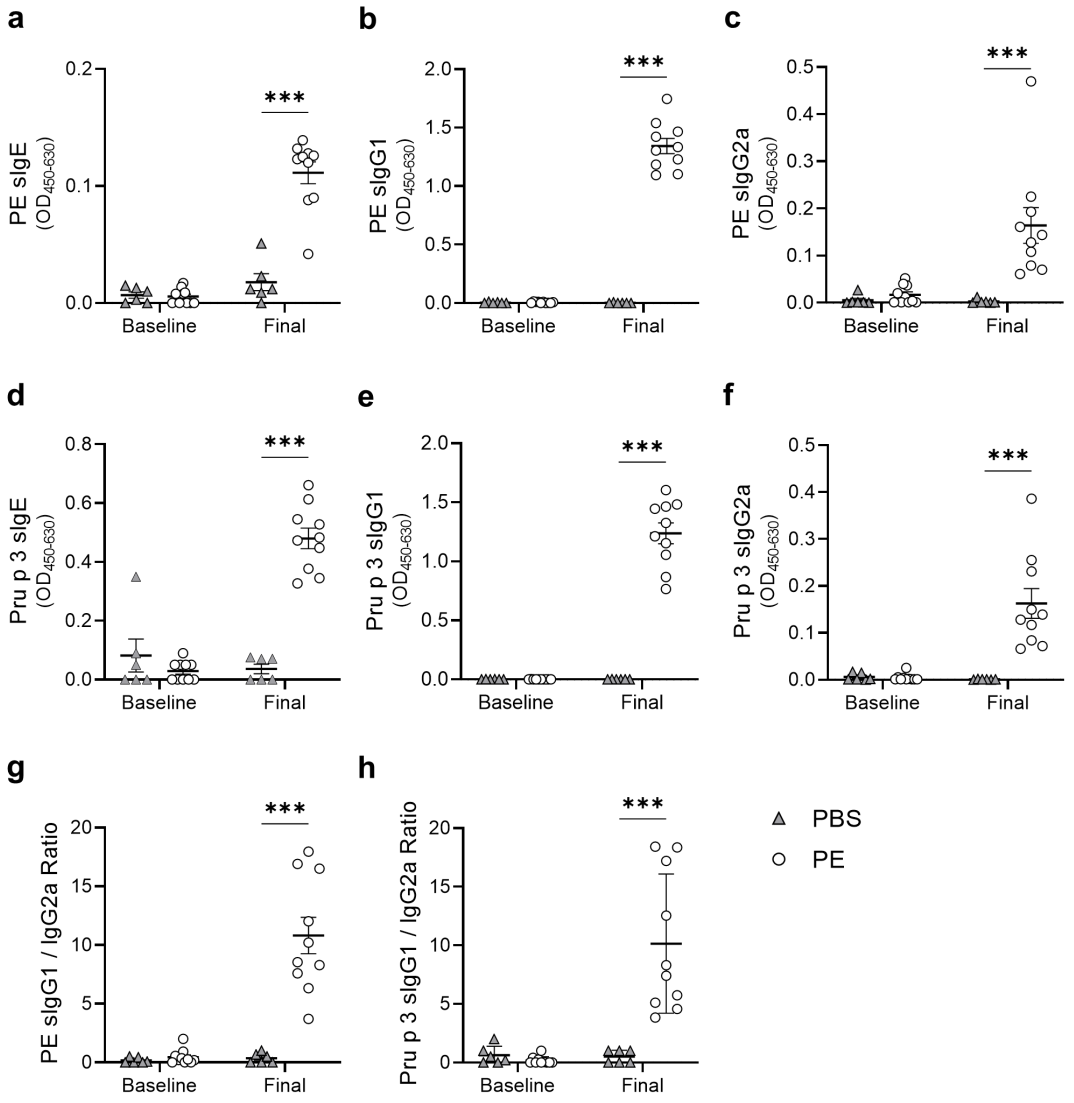

Figure 33: Induction of humoral immune response by PE.
Specific antibody responses were measured in the serum of the mice before sensitization (Baseline) and after provocation (Final). Levels of PE-specific (a) IgE, (b) IgG1 and (c) IgG2a, as well as Pru p 3-specific (d) IgE, (e) IgG1 and (f) IgG2a were analyzed. Ratio of (g) PE-specific and (h) Pru p 3-specific IgG1 to IgG2a were determined for baseline and final samples. n = 6-10; data presented as combination of two experiments performed under identical settings; ***$p < 0.001$. Data published in Steigerwald *et al.* 2023.

In line with the increased levels of specific antibodies in the serum, levels of mMCPT-1 were significantly increased in the serum of PE-immunized mice (Figure 34a). In addition, Pru p 3- and PE-specific IgG immune response was corroborated by immunoblot (Figure 34b). In the serum samples taken after provocation, the PE-immunized mice showed antibody binding mainly against nPru p 3 but also to other yet unidentified proteins in the PE.

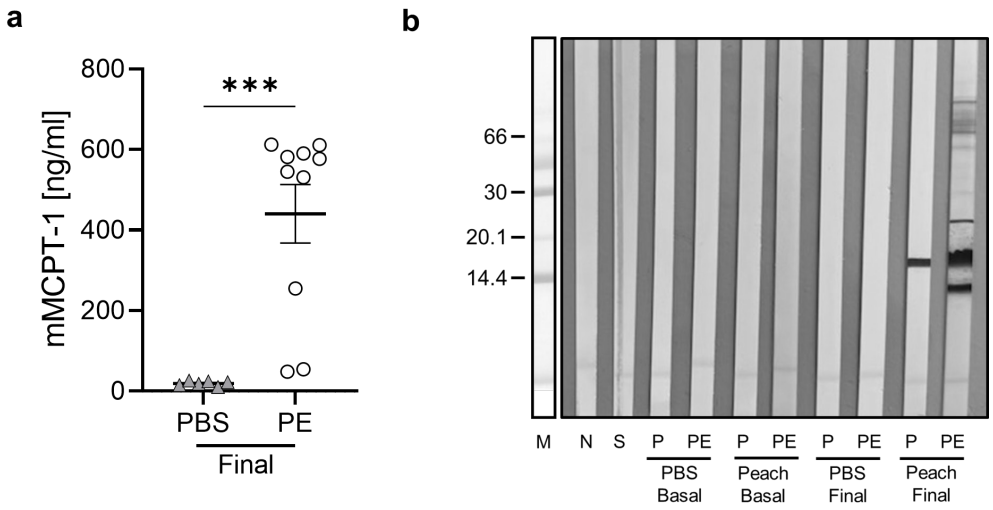

Figure 34: mMCPT-1 levels and IgG reactivity of mouse serum.
Levels of (a) mMCPT-1 were examined in the serum of the mice after provocation (Final). (b) Peach peel extract (PE) and nPru p 3 (P) were subjected to SDS-PAGE followed by immunoblot analysis using pooled serum from mice of the experimental groups before sensitization (Basal) and after provocation (Final). n = 6-10, data presented as combination of two experiments performed under identical settings; ***$p < 0.001$. Data published in Steigerwald *et al.* 2023.

Furthermore, total levels of Ig were measured in the serum and PE-immunized mice showed significantly increased levels of total IgE and IgG after provocation (Figure 35a, b). In comparison, total IgA levels were significantly reduced in peach allergic mice, when compared to control mice and baseline values (Figure 35c).

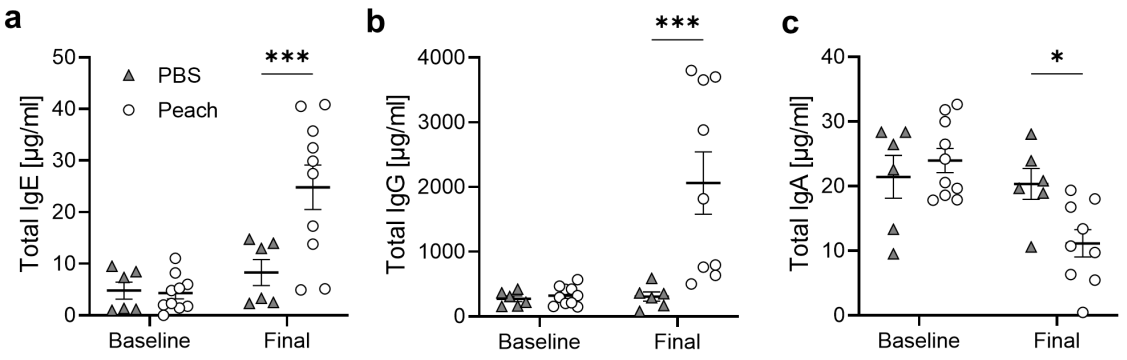

Figure 35: Total immunoglobulin levels measured in the serum of peach allergic mice.
Levels of (a) total IgE, (b) total IgG and (c) total IgA were analyzed in the serum before sensitization (Baseline) and after provocation (Final). n = 6-10, data presented as combination of 2 experiments performed under identical settings; *$p < 0.05$; ***$p < 0.001$. Data published in Steigerwald *et al.* 2023.

Analysis of the cytokine secretion after re-stimulation of splenocytes with PMA and Ionomycin revealed significantly enhanced levels of Th2-cytokines IL-4 and IL-13 after PE-exposure (Figure 36). In comparison, levels of IL-5 remained unaffected by PE-immunization and levels of IFNγ tended to be decreased in the peach allergic mice compared to the control group.

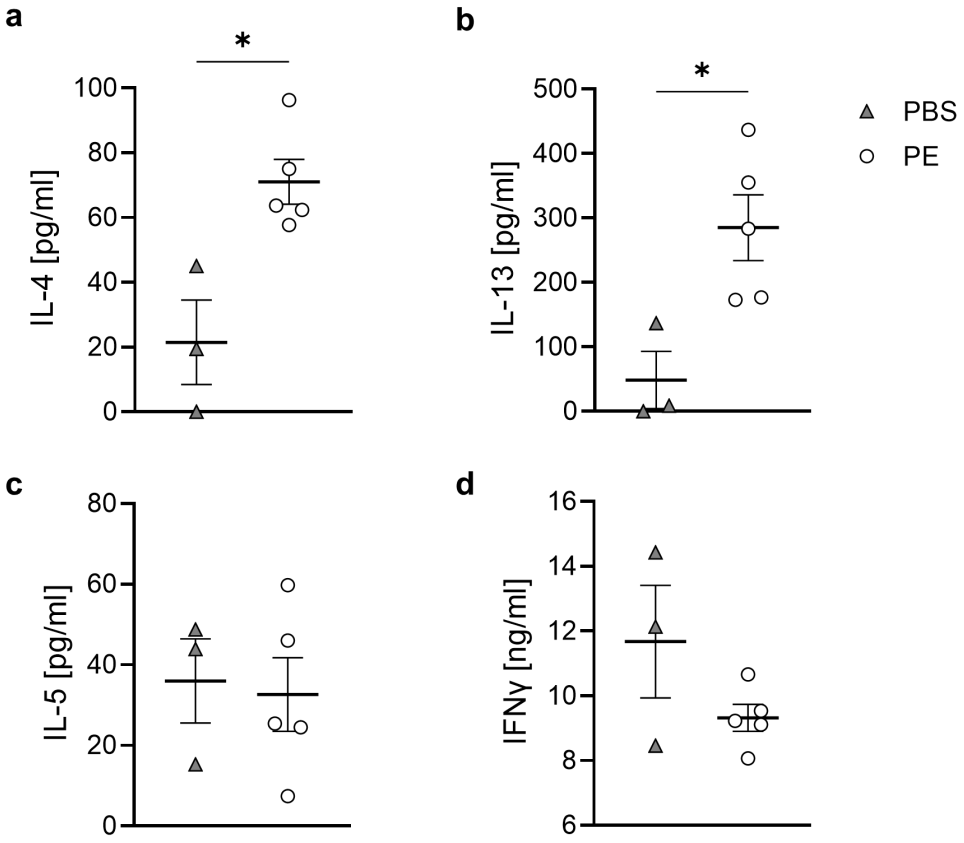

Figure 36: Measurement of cytokine secretion in peach allergic mice.
Levels of (a) IL-4, (b) IL-13, (c) IL-5 and (d) IFNγ were measured by ELISA in the supernatant of splenocytes re-stimulated with PMA and Ionomycin. n = 3-5, *$p < 0.05$. Data published in Steigerwald *et al.* 2023.

To successfully establish FA in a mouse model, a systemic reaction but also involvement of the intestinal tract is crucial. Thus, physiological alterations and infiltrating T/B cells and granulocytes of the LP were analyzed to characterize the FA-related inflammatory reaction in the gut.

Measurement of the small and large intestine length revealed a significant enlargement of the small intestine in the PE-immunized mice and a tendency could be observed for the large intestine (Figure 37).

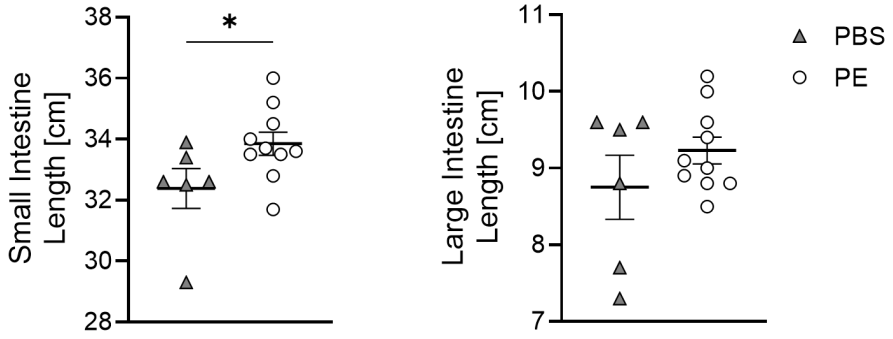

Figure 37: Measurement of intestine length in peach allergic mice.
Length of small and large intestine were measured after provocation and euthanasia of the mice. n = 6-10, data presented as combination of 2 experiments performed under identical settings. *$p < 0.05$. Data published in Steigerwald *et al.* 2023.

Furthermore, flow cytometry analysis of infiltrating immune cells in the small intestine LP, reported as the intestinal site where FA sensitization and inflammatory responses take place (Ali et al. 2020), revealed induction of local immune responses in peach allergic mice (Figure 38). This was characterized by an enhanced frequency of T cells ($CD3^+$ cells), including Th cells ($CD4^+$ cells) and Tregs ($CD4^+$ $Foxp3^+$ cells), while CTLs ($CD8^+$ cells) were just slightly enhanced and no difference was observed in the frequency of B cells ($CD19^+$ cells). Moreover, the frequency of neutrophils and cDCs was significantly enhanced in the mice immunized with PE when compared to the PBS-treated controls.

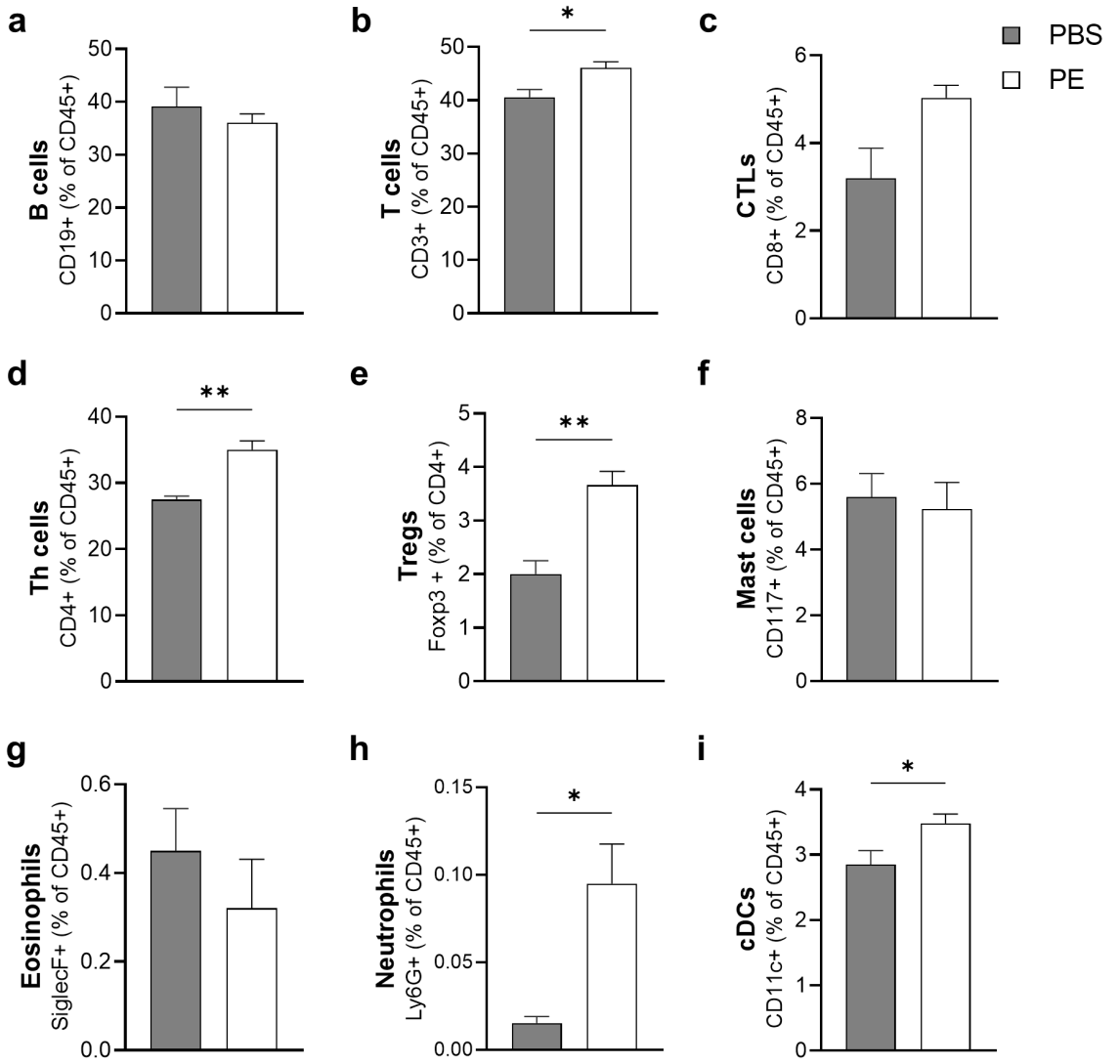

Figure 38: Infiltrating immune cells in the small intestine lamina propria of peach allergic mice.

The small intestine was enzymatically treated and isolated LP immune cells were analyzed via FACS. (a) B cells, (b) T cells, (c) cytotoxic T cells (CTLs), (d) T helper cells (Ths), (e) regulatory T cells (Tregs), (f) mast cells, (g) eosinophils, (h) neutrophils and (i) conventional dendritic cells (cDCs). n = 3-5; *$p < 0.05$; **$p < 0.01$. Data published in Steigerwald *et al.* 2023.

Interestingly, eosinophils and mast cells were not enhanced in the PE-immunized mice, however, local activation of mast cells in the intestine was indicated by significantly increased mMCPT-1 levels in the supernatant of intestinal homogenates when mice were exposed to PE (Figure 39a). Additionally, measurement of local antibody concentrations in the small intestine homogenates revealed increased levels of total IgE (Figure 39b) and IgG (Figure 39c) but not IgA (Figure 39d) when the mice were immunized with PE.

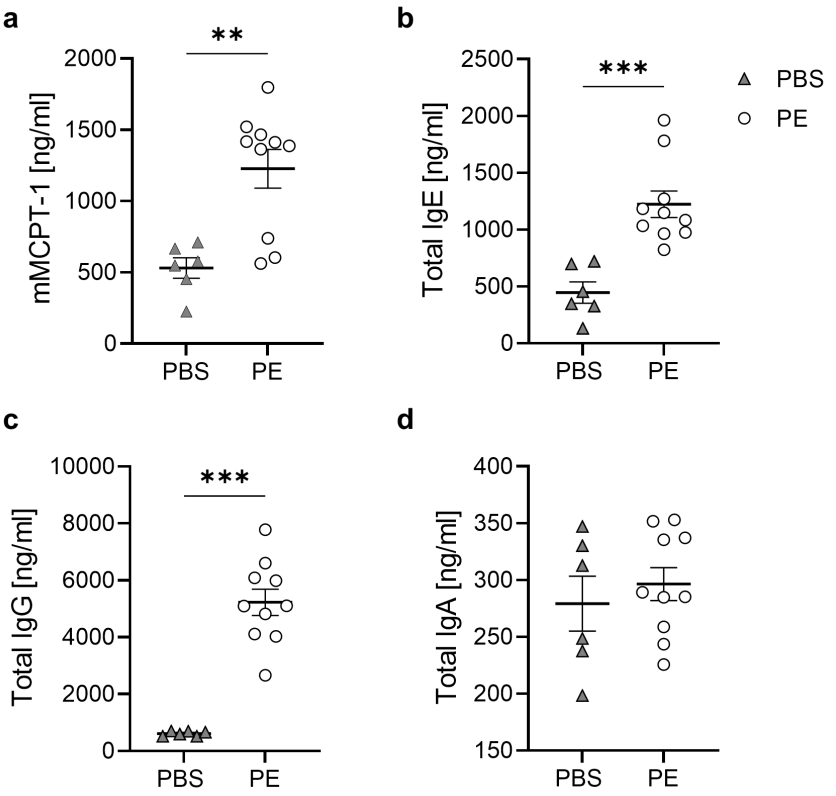

Figure 39: mMCPT-1 and total Ig levels in the intestine of peach allergic mice.
Levels of (a) mMCPT-1, (b) total IgE, (c) total IgG and (d) total IgA were determined in the supernatant of intestinal homogenates by ELISA. n = 6-10, data presented as combination of 2 experiments performed under identical settings; $^{**}p < 0.01$; $^{***}p < 0.001$. Data published in Steigerwald *et al.* 2023.

In summary, these results indicated that administration of PE induced systemic allergen-specific immune reactions, as well as local immune responses in the intestinal tract. Thus, the model was chosen for induction of peach allergy in mice and in the following to perform the pectin intervention experiments.

4.4 Pectin intervention in peach allergic mice using apple-derived HMP

The experiments shown in section 4.2 revealed a strong modulatory effect of pectin supplementation and especially HMP on the immune response and gut microbiota composition in healthy mice. The effects observed showed to be dose-dependent and tended to be stronger when higher amounts of 15% pectin, especially HMP were supplemented in the diet.

As this study aimed to investigate the role of pectin on FA, in particular peach allergy, dietary intervention was performed in peach allergic mice. Due to the observed strong effects of HMP supplementation to healthy mice, this pectin was chosen for the intervention study and 15% were supplemented to the diet of the allergic mice.

4.4.1 HMP intervention does not suppress allergy-related clinical signs

The effect of HMP on peach allergic mice was examined by dietary intervention of 15% HMP either before start of the allergic sensitization (“primary-prophylactic” approach) or after sensitization (“secondary-prophylactic” approach) (refer to Figure 8). Mice of the allergic control and the PBS control were fed a control diet containing 20% cellulose. Body weight, food consumption and general health status of the mice was monitored throughout the experiment (Figure 40). The data showed that dietary intervention with HMP in peach allergic mice did not affect the total body weight gain (Figure 40a) or total food intake (Figure 40b) as all treatment groups had gained weight during the experiment. A slight but not significantly enhanced body weight gain was observed in the allergic control group in comparison to the PBS control group.

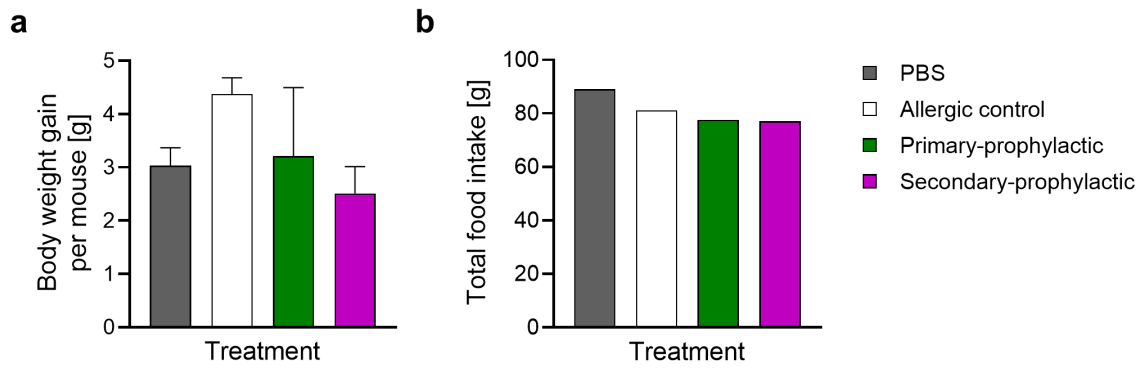

Figure 40: Body weight gain and total food intake after 15% HMP intervention.
Mice were monitored closely in regard of (a) body weight gain and (b) food intake during the period of the pectin intervention experiment. n = 3-5.

Core body temperature of the mice was monitored 15 min before and up to 30 min after oral exposure (Figure 41) or provocation (Figure 42a,b), whereas no decrease of body temperature was observed after threefold oral exposure.

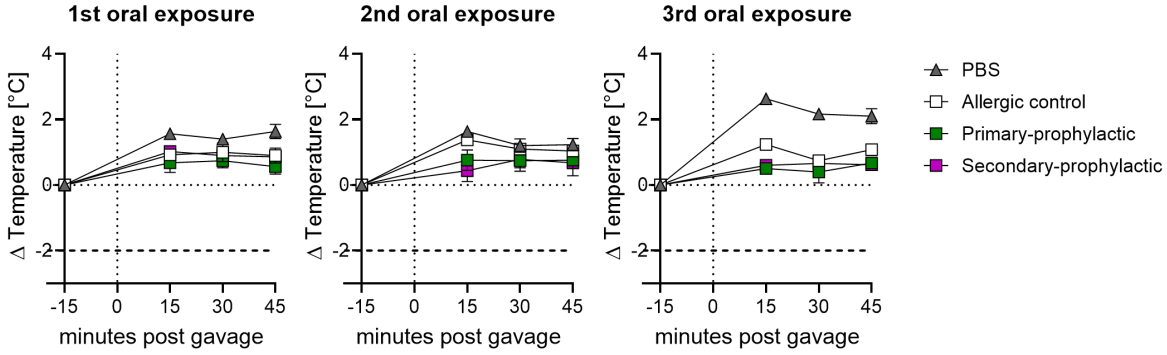

Figure 41: Body temperature after oral exposure.
Body temperature of the mice was measured 15 min before and up to 45 min after each oral exposure to PE or PBS. n = 3-5

A significant temperature drop was measured 15 min after provocation in all PE-immunized mice, showing no difference between the pectin intervention and the allergic control (Figure 42a,b). Additionally, allergy-related clinical signs were examined in a blinded manner as described before (Table 10), after each oral exposure and the provocation. First allergy-related signs could be observed after the 2nd oral exposure, including calm behavior or slightly ruffled fur, whereas the strongest symptoms were observed after provocation with an average score of

around seven in the allergic control. A slight but not significant reduction of the symptom score was detected in both pectin intervention groups, when compared to the allergic control (Figure 42c).

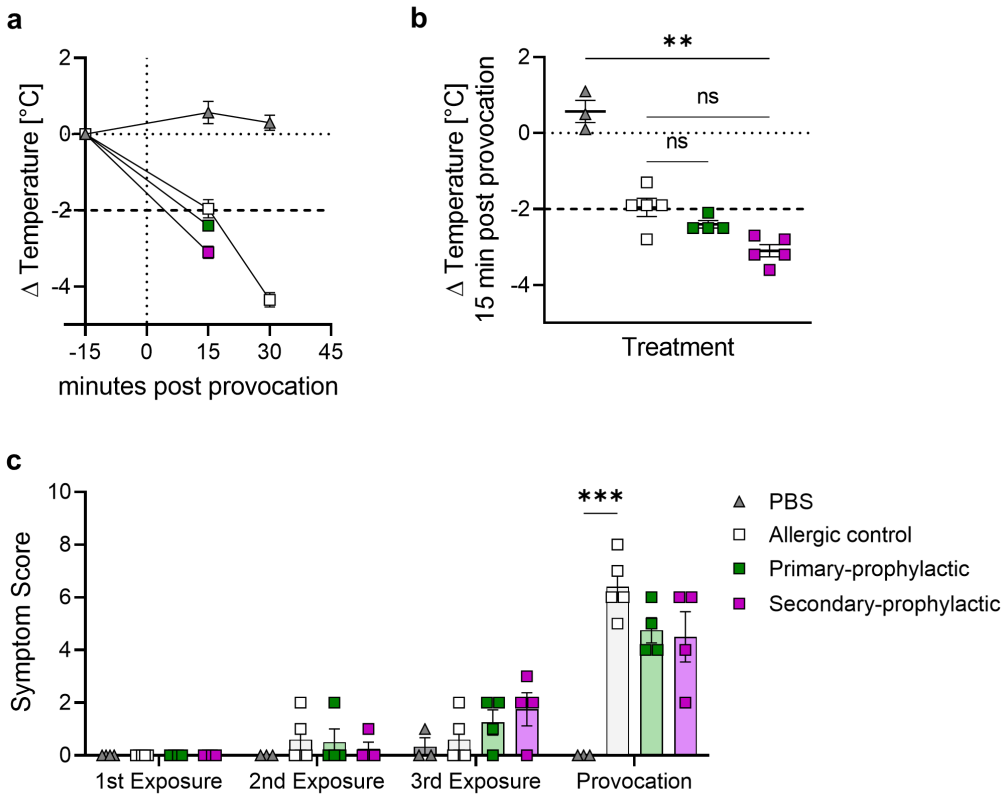

Figure 42: Allergy-related clinical signs after HMP intervention and provocation.
(a) Body temperature of the mice was measured before (-15 min; baseline) and up to 30 min after provocation. (b) Body temperature of individual mice 15 min after provocation and (c) symptom score after oral exposure or provocation. n = 3-5; $^{**}p < 0.01$; $^{***}p < 0.001$.

4.4.2 Modulation of immune response by HMP intervention

The humoral immune response was analyzed in the serum of mice after provocation (Figure 43). Measurement of PE- and Pru p 3-specific antibody levels revealed a significant increase of sIgE and sIgG1 in the allergic control mice compared to the PBS control. Both pectin intervention approaches significantly decreased the levels of sIgE and primary-prophylactic HMP intervention significantly decreased levels of sIgG1 compared to the allergic control. In contrast, secondary-prophylactic HMP intervention induced a significant increase of Pru p 3-sIgG2a and slightly enhanced

levels of PE-sIgG2a. Evaluation of the ratio of PE- or Pru p 3-specific IgG1 to IgG2a revealed a significant increase of the ratio in the allergic control which was decreased to baseline levels by both pectin interventions (Figure 43g,h).

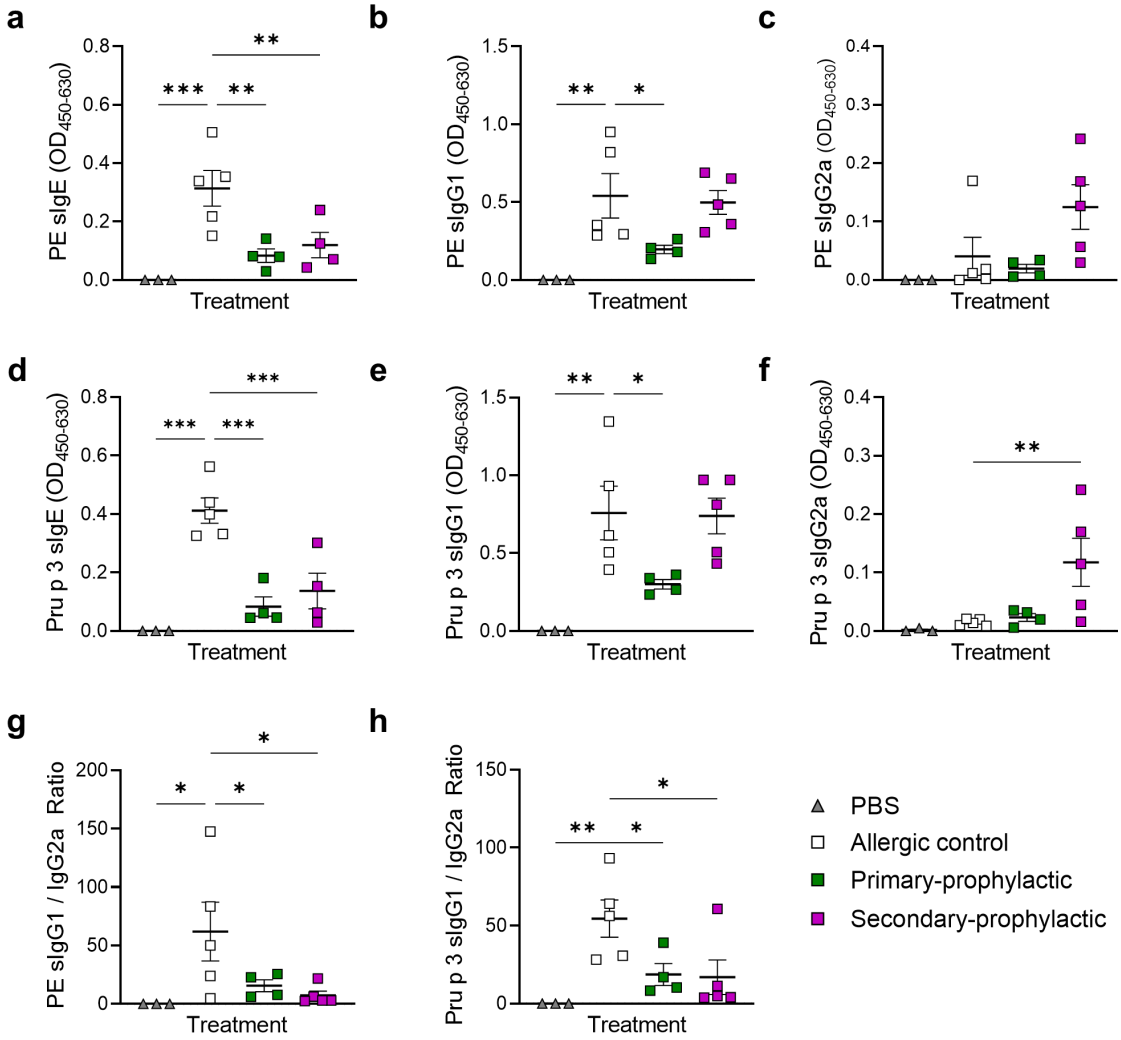

Figure 43: Allergen-specific antibody response in the serum after HMP intervention.
PE- and Pru p 3-specific antibody response were examined in the serum of the mice after provocation. Levels of PE-specific (a) IgE, (b) IgG1 and (c) IgG2a, as well as Pru p 3-specific (d) IgE, (e) IgG1 and (f) IgG2a were analyzed by ELISA. Ratio of (g) PE-specific and (h) Pru p 3-specific IgG1 to IgG2a was determined. n = 3-5; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

In addition, levels of mMCPT-1 were significantly enhanced in the serum of the allergic control group compared to the PBS control (Figure 44a). Primary-prophylactic pectin intervention led to a significant decrease of these levels compared to the allergic control, whereas no effect was observed by secondary-

prophylactic HMP intervention. Levels of total IgE were significantly increased in the serum of the allergic control mice compared to the PBS control, whereas primary-prophylactic but not secondary-prophylactic pectin intervention could significantly reduce these levels in the serum (Figure 44b). A significant increase of total IgG was observed only in the allergic control when compared to the PBS control group. However, mice of the secondary-prophylactic pectin intervention showed slightly lower total IgG levels than the allergic control mice (Figure 44c). Furthermore, levels of total IgA were strongly decreased in the allergic control as it was observed before in the establishment of the peach allergy mouse model (refer to Figure 35). In comparison, both pectin interventions restored the levels of total IgA, comparable to control group values (Figure 44d). Examination of the ratio between total IgG to total IgE revealed a decreased ratio in the allergic control, whereas primary-prophylactic but not secondary-prophylactic pectin intervention significantly reversed this effect (Figure 44e).

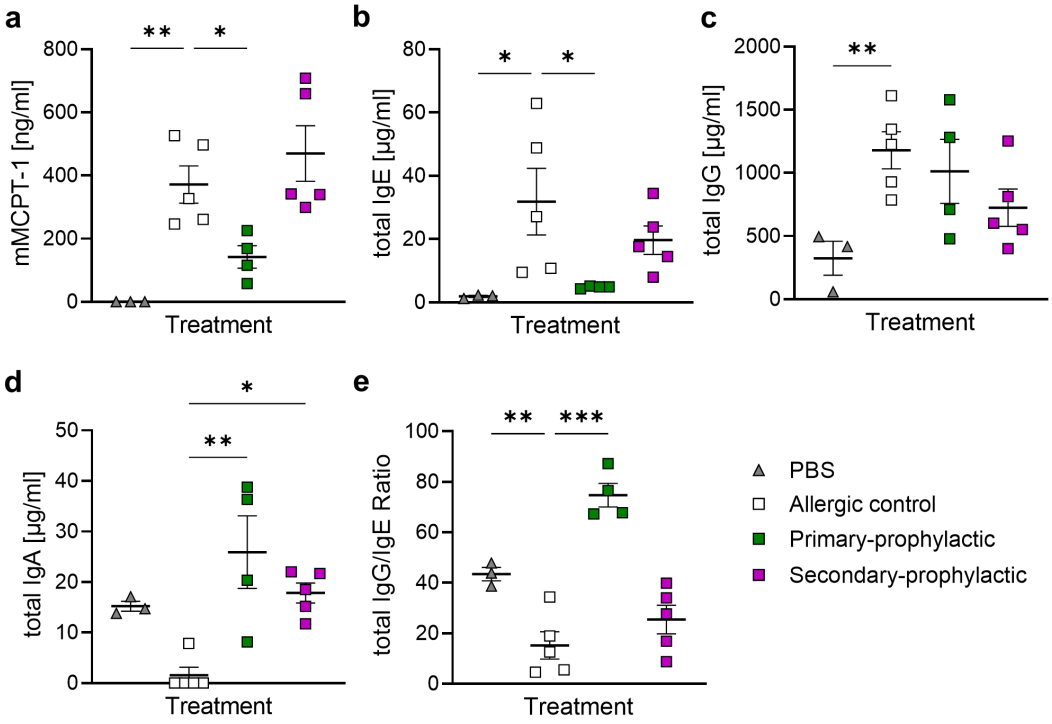

Figure 44: mMCPT-1 levels and total immunoglobulin levels after HMP intervention.
Levels of (a) mMCPT-1, (b) total IgE, (c) total IgG, (d) total IgA as well as (e) the ratio of total IgG to total IgE were examined in the serum of the mice after provocation. n = 3-5; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Analysis of cytokine secretion after re-stimulation of splenocytes with PMA and Ionomycin revealed no effect on the levels of IL-4 (Figure 45a) whereas levels of IL-13 were significantly enhanced in the allergic control, when compared to primary-prophylactic HMP intervention (Figure 45b). Levels of IL-5 were enhanced in two out of five mice of the allergic control group, whereas HMP intervention induced no effects (Figure 45c). Similarly, no significant effect on IFNγ levels was observed after PE-sensitization or HMP intervention (Figure 45d).

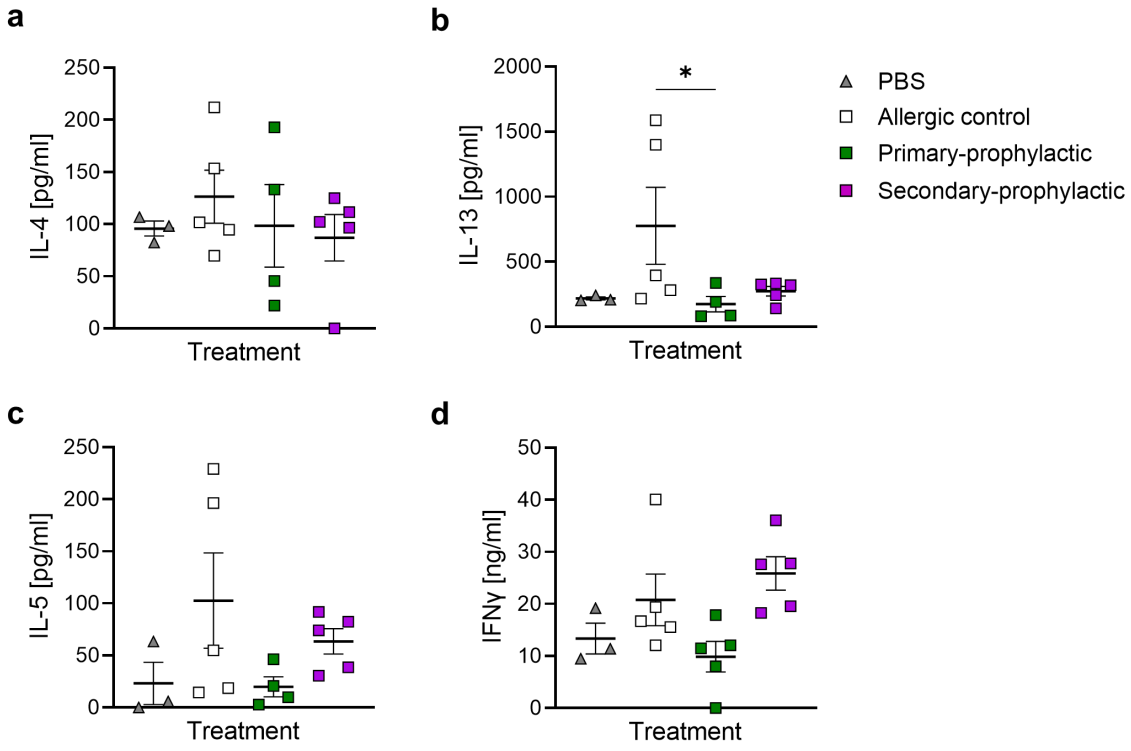

Figure 45: Measurement of cytokine secretion after HMP intervention.
Levels of a) IL-4, b) IL-13, c) IL-5 and d) IFNγ were measured in the supernatant of splenocytes re-stimulated with PMA and Ionomycin by ELISA. n = 3-5, *$p < 0.05$

4.4.3 HMP affects gut microbiota composition in peach allergic mice

The effect of HMP on the gut microbiota composition was evaluated by 16S rRNA analysis of feces samples collected at different time points (Figure 47). The microbiota analysis in this work package was done in collaboration with Dr. Csaba Miskey (Division of Medical Biotechnology; Paul-Ehrlich-Institut, Langen, Germany), who performed sequencing of the DNA library and Dr. Oleg Krut (Microbiological

Safety Section; Paul-Ehrlich-Institut, Langen, Germany), who did the bioinformatic analysis of the results.

HMP intervention (primary- and secondary-prophylactic) significantly reduced the microbial richness, calculated as Chao1 index (non-parametric, abundance-based estimator of species richness (Kim et al. 2017)), in the fecal samples after 4 days of pectin intervention (day -2 for primary-prophylactic intervention or day 16 for secondary-prophylactic intervention). The Chao1 index of the allergic control was not changed during the period of the experiment. Unexpectedly, also the microbial richness of the PBS control was decreased from day -2 on.

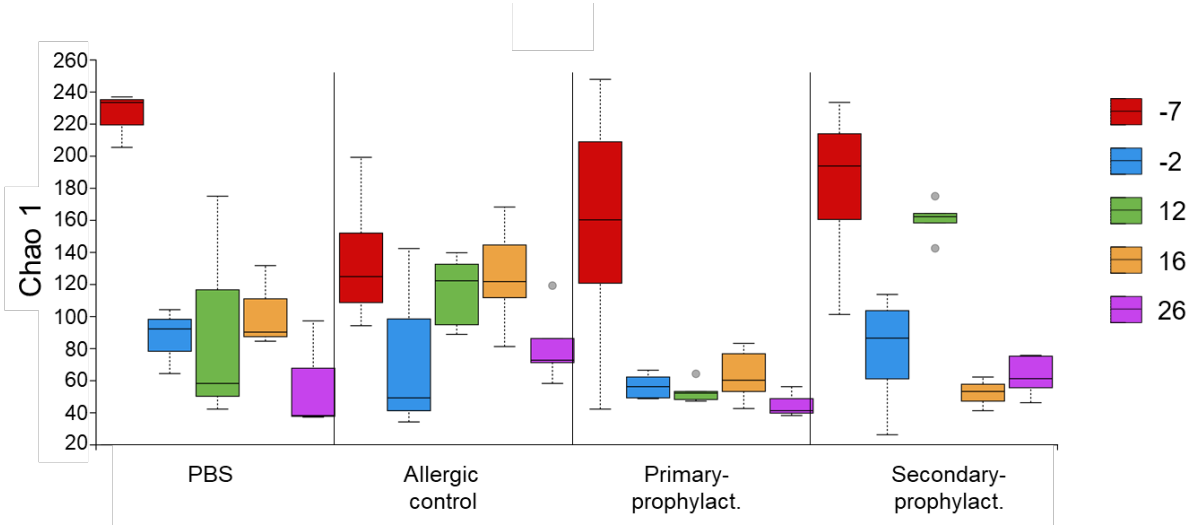

Figure 46: Effect of HMP intervention on microbial richness.
Microbial content was analyzed in feces samples and richness was calculated as Chao1 index. The data were generated and provided by Dr. Csaba Miskey and Dr. Oleg Krut from the Paul-Ehrlich-Institut, Langen, Germany.

Furthermore, both HMP intervention approaches led to a substantial shift of the bacterial composition towards *Bacteroides*, which could be observed 4 days after start of the pectin diet (day -2 for primary-prophylactic, day 16 for secondary-prophylactic approach) (Figure 47). Interestingly, this bacterial genus was slightly reduced in the allergic control after sensitization (day 12). Contrary to the increase of *Bacteroides* after start of the pectin diet, *Parabacteroides* decreased in these groups and increased in the allergic control. Additionally, frequency of *Akkermansia*

was strongly reduced after start of pectin intervention, whereas the abundance stayed constant in the allergic control group.

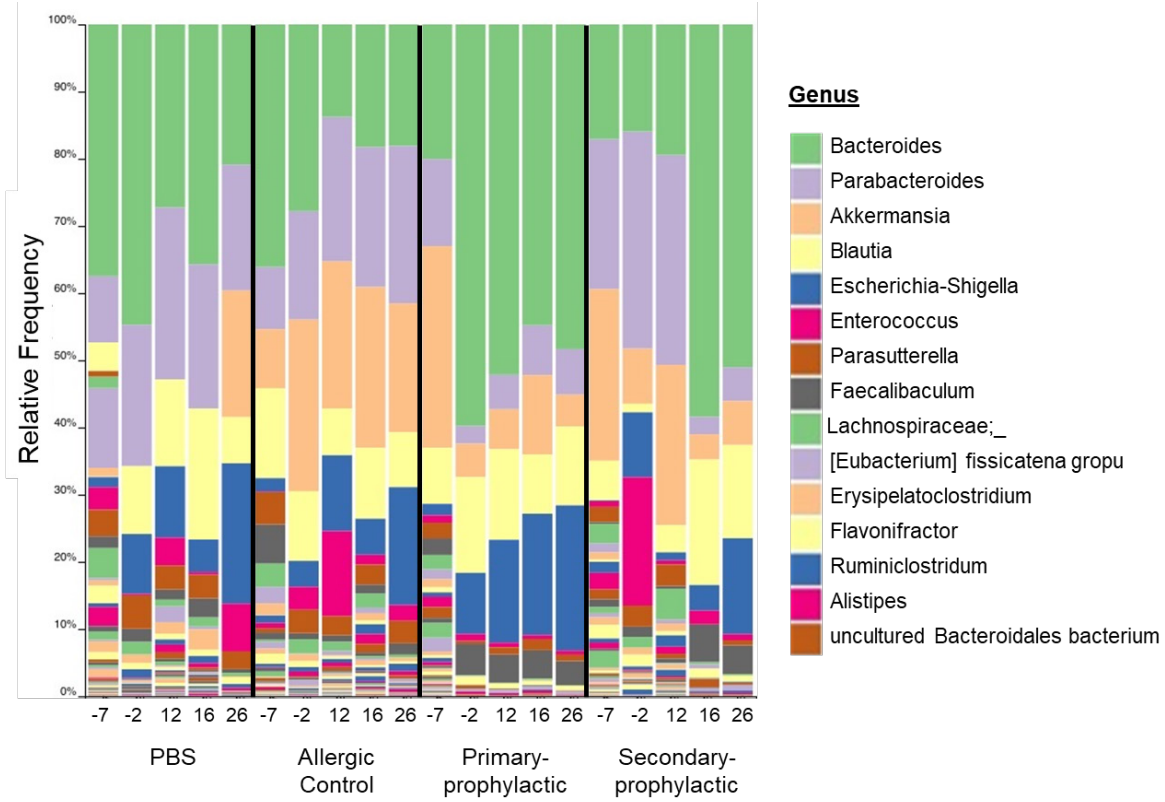

Figure 47: Modulation of gut microbiota composition by HMP intervention.
Relative frequency of bacterial genera was examined in feces samples of the mice at five different time points by 16S-rRNA analysis. Sequencing of the isolated DNA samples was done by Dr. Csaba Miskey and bioinformatic analysis was performed by Dr. Oleg Krut from the Paul-Ehrlich-Institut, Langen, Germany. n = 3-5

4.4.4 Modulation of intestinal immune response by HMP intervention

Besides modulation of the gut microbiome, physiological alterations of the intestinal tract as consequence of the pectin intervention were assessed. Results showed that the length of the small intestine was slightly enlarged in the allergic control mice, which was reduced by the primary-prophylactic pectin intervention (Figure 48a). The length of the large intestine was significantly increased in both pectin intervention groups compared to the allergic control (Figure 48b). A similar effect was observed on caecum size, which was slightly smaller in the mice of the allergic control but was

significantly increased after pectin intervention compared to the allergic control (Figure 48c).

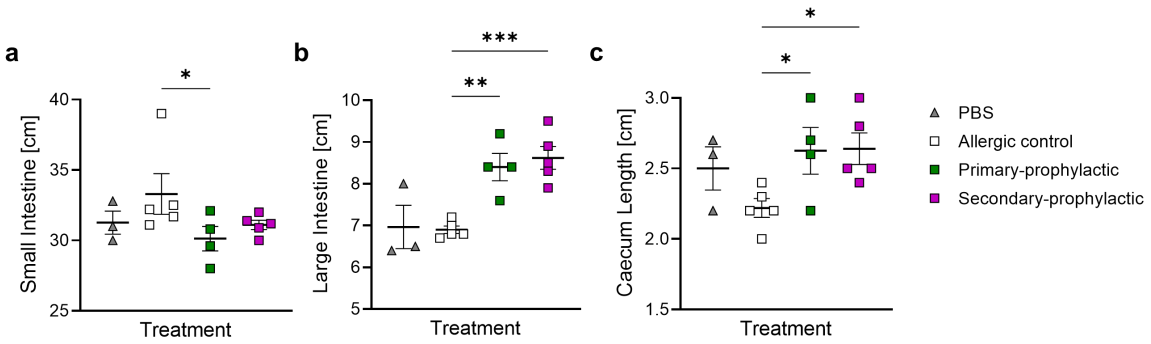

Figure 48: Length of small intestine, large intestine and caecum after HMP intervention. Length of the (a) small intestine, (b) large intestine and (c) caecum was measured after provocation and euthanasia of the mice. n = 3-5; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Furthermore, levels of mMCPT-1, total IgE, total IgG and total IgA were analyzed in the intestinal homogenates (Figure 49).

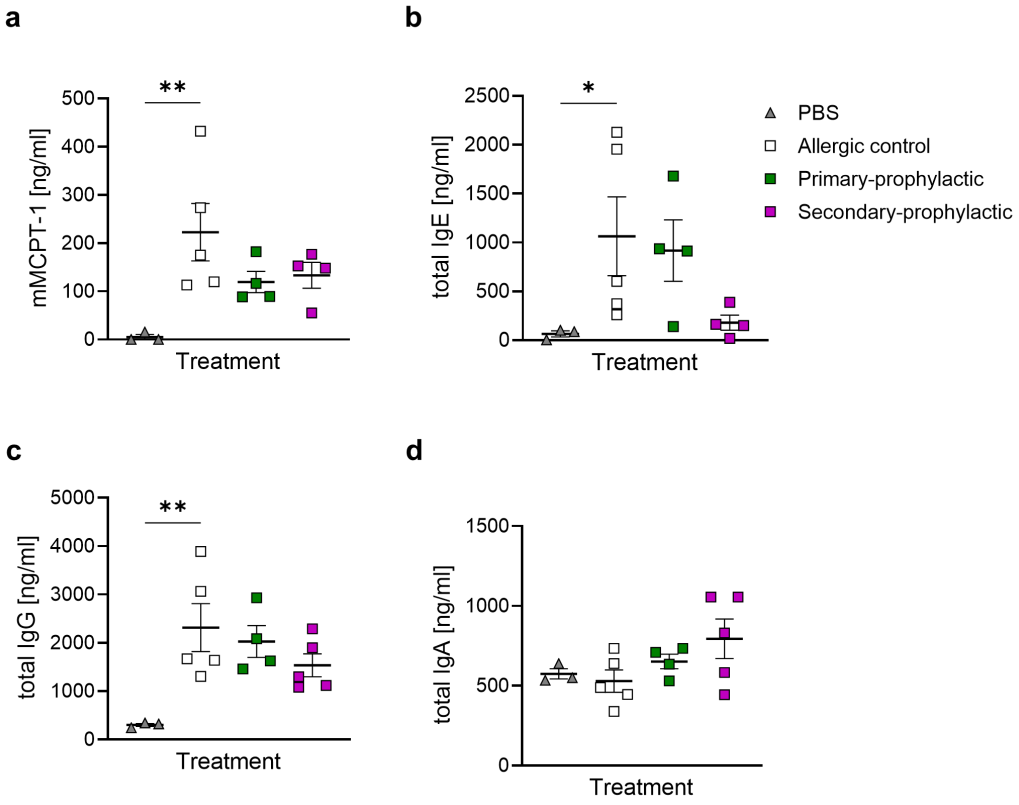

Figure 49: mMCPT-1 and total Ig levels in intestinal homogenates after HMP intervention. Levels of (a) mMCPT-1, (b) total IgE, (c) total IgG and (d) total IgA were analyzed in the supernatant of intestinal homogenates of the mice after provocation via ELISA. n = 3-5; **p* < 0.05; ***p* < 0.01.

mMCPT-1 levels were significantly increased in intestinal homogenates of the allergic mice and slightly but not significantly decreased by both HMP interventions (Figure 49a). Levels of total IgE were enhanced in the allergic control but slightly reduced by secondary-prophylactic HMP intervention (Figure 49b). In contrast, total IgG levels were enhanced in the allergic control group, which could tendentially be observed in both pectin intervention groups (Figure 49c). Levels of total IgA showed no differences between the PBS or allergic control as well as the pectin interventions (Figure 49d).

To investigate, whether dietary intervention with HMP exerts effects on the immune cells, infiltrating cells of the small and large intestine LP were analyzed by FACS (Figure 50). The data showed a significant decrease of mast cells and macrophages in the small intestine in both HMP intervention approaches, compared to the allergic control. A similar tendency was observed in the large intestine by primary-prophylactic HMP intervention. Frequencies of eosinophils and neutrophils were not significantly affected in the large or small intestine. cDCs were significantly decreased in the small intestine of the allergic control mice compared to the PBS control, which was also tendentially observed in the HMP intervention groups.

In addition, frequencies of T and B cells as well as subpopulations were evaluated by flow cytometry (Figure 51). With secondary-prophylactic intervention approach, HMP induced increased levels of B cells in the small intestine. An increase of T cells was observed in the small intestine of the allergic control, that was reduced to baseline levels after both HMP interventions. Interestingly, only primary-prophylactic HMP intervention reduced CTLs in the small intestine, whereas both HMP interventions tended to reduced CTL frequencies in the large intestine. Furthermore, HMP intervention led to increased levels of Th cells in the small intestine. No significant effect on the frequency of Tregs was observed between the treatment groups.

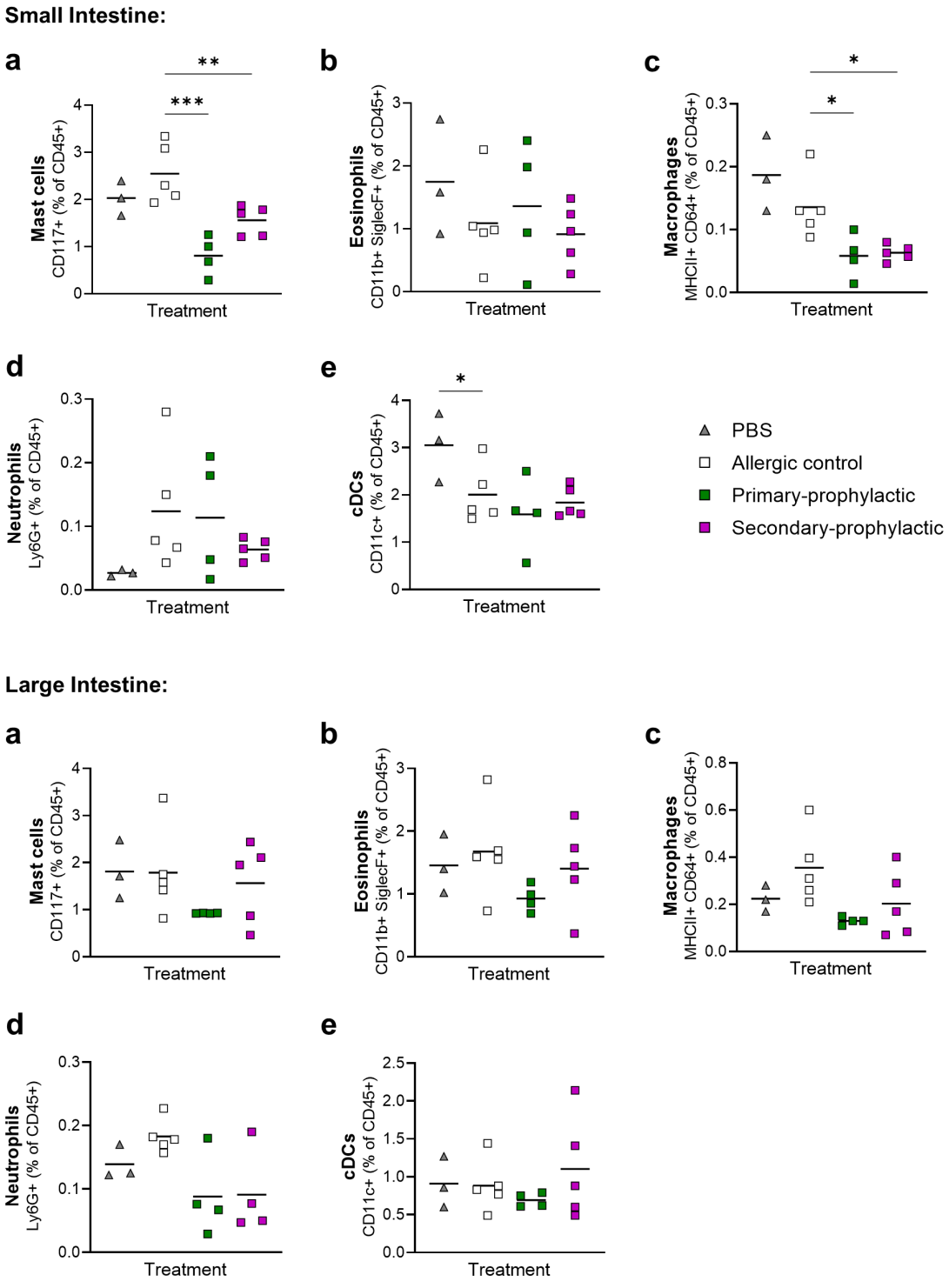

Figure 50: Analysis of infiltrating granulocytes in the lamina propria of small and large intestine.

Small and large intestine were enzymatically treated and isolated LP immune cells were analyzed via FACS. (a) Mast cells, (b) eosinophils and (c) macrophages, (d) neutrophils and (e) cDCs. n = 3-5; *$p < 0.05$; ***$p < 0.001$.

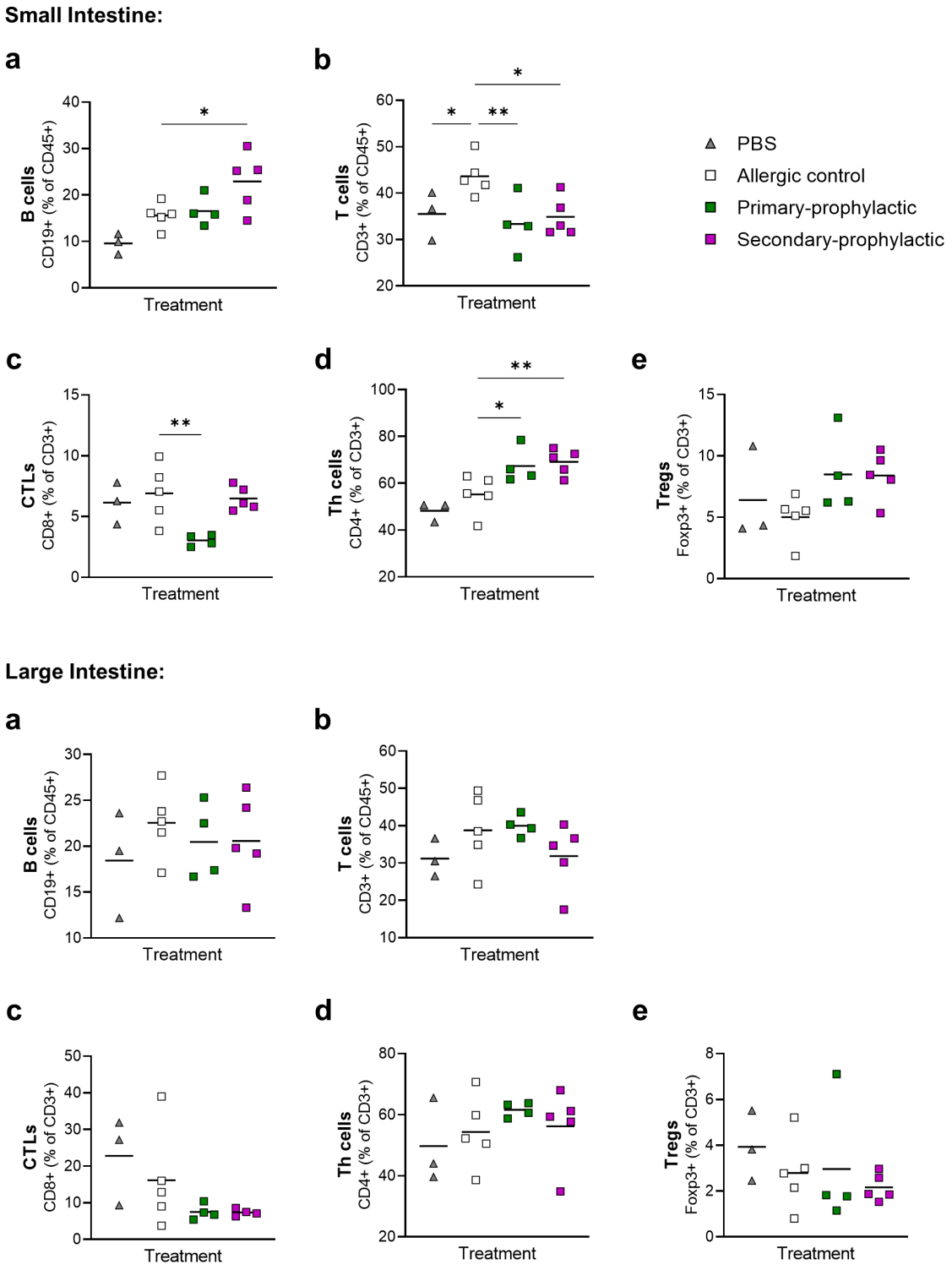

Figure 51: Analysis of infiltrating T and B cells in the lamina propria of small and large intestine.
Isolated infiltrating cells of the LP of small and large intestine were analyzed by FACS. (a) B cells, (b) T cells, (c) cytotoxic T cells (CTLs), (d) T helper cells (Th cells) and (e) regulatory T cells (Tregs). n = 3-5; *$p < 0.05$; **$p < 0.01$.

In summary, the results indicated that dietary intervention with 15% HMP in peach allergic mice exerted substantial effects on the allergy-related immune response, the gut microbiota composition as well as the intestinal immune cells, especially in the small intestine. The modulatory effect of HMP was mainly characterized by e.g suppression of PE- and Pru p 3-specific antibody response as well as reduced levels of total IgE and mMCPT-1. In contrast, levels of total IgA were elevated after HMP intervention and intestinal microbiota was modulated by promotion of especially *Bacteroides*. Furthermore, HMP intervention seemed to reduce the frequencies of mast cells, macrophages and neutrophils in the intestine. However, slight differences in the exerted effects were visible, comparing both intervention approaches. This indicates that the immune-modulatory effects of HMP in peach allergic mice also depend on the duration of supplementation as well as the chosen time point for pectin intervention during the sensitization and manifestation phases of FA.

4.5 Comparison of HMP and LMP intervention in peach allergic mice

The results achieved in this study so far, indicated that structurally different pectins exert distinct modulatory effects on the immune response and composition of the gut microbiota in healthy mice (see section 4.2). HMP was further evaluated in peach allergic mice, showing clear effects on the humoral and cellular immune response as well as on the gut microbiota (see section 4.4). Dietary intervention with HMP could e.g. decrease the levels of allergen-specific IgE in the allergic mice and suppress activation of mast cells. However, considering the structural differences among pectins, it is not known whether LMP might as well promote any beneficial immune-modulatory effects in allergic mice. Therefore, the results obtained with HMP should be confirmed and the effects of pectin intervention comparing HMP and LMP were evaluated in peach allergic mice.

4.5.1 Pectin intervention does not suppress development of symptoms

Pectin intervention in peach allergic mice was performed with HMP and LMP (each 15%) as primary-prophylactic (start of dietary intervention before sensitization, day -7) or secondary-prophylactic (start of dietary intervention after sensitization, day 12) approach (refer to Figure 8). The mice were closely monitored in regard of body weight, food consumption and general health status (Figure 52).

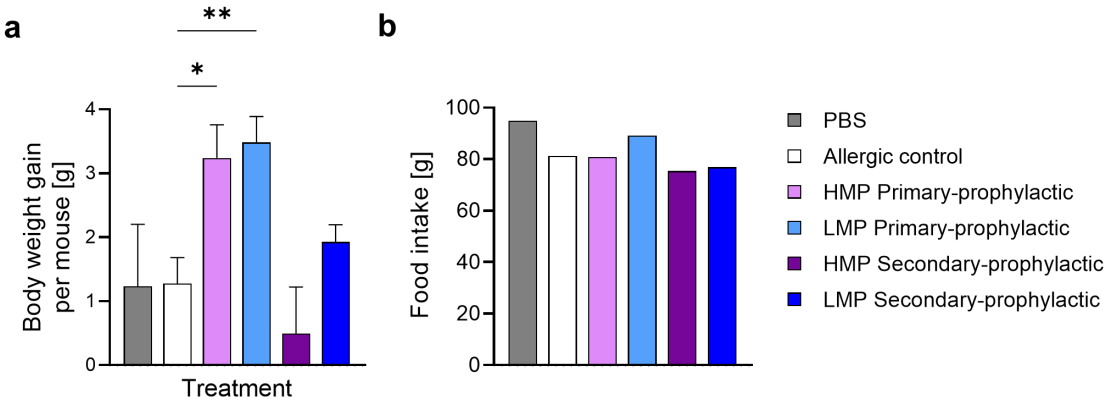

Figure 52: Body weight gain and food intake during pectin intervention with HMP or LMP. Mice were monitored closely in regard of (a) body weight gain and (b) food intake during the pectin intervention period with HMP or LMP. n = 3-5; *$p < 0.05$; **$p < 0.01$.

All groups gained weight during the intervention experiment (Figure 52a). However, mice that received pectin (LMP or HMP) as primary-prophylactic intervention gained significantly more weight compared to the peach allergic control, whereas there was no difference in the total food intake (Figure 52b).

Body temperature and clinical signs of the mice were recorded 15 min before and up to 45 min after each oral exposure (Figure 53) or provocation (Figure 54). The body temperature of all groups stayed stable after each of the three oral exposures.

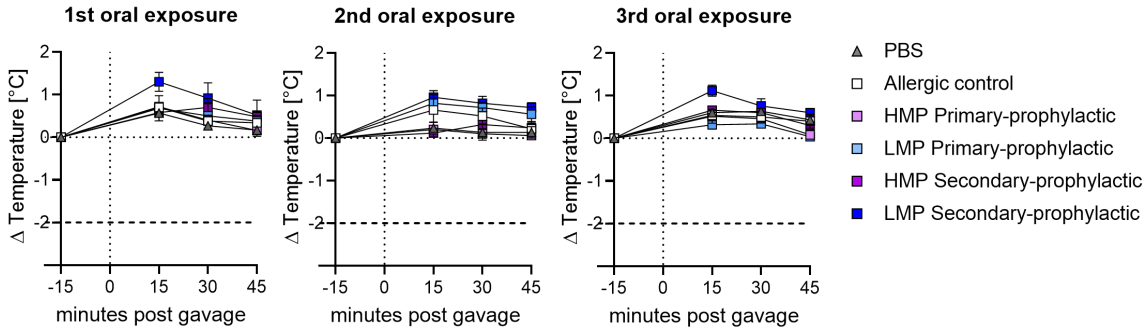

Figure 53: Body temperature after oral exposure in pectin intervention experiment.
Body temperature of the mice was measured 15 min before and up to 45 min after each oral exposure to PE or PBS. n = 3-5

All PE-immunized mice developed a strong drop in body temperature by an average of 3°C 15 min after i.p. provocation with no observable differences between the pectin intervention groups and the allergic control (Figure 54a,b). Clinical signs slightly increased after oral exposure as denoted by the symptom scores, but were highly enhanced (around score six out of eight) after the provocation (Figure 54c). Interestingly, pectin intervention could not avoid but primary-prophylactic approach slightly reduced the development of clinical signs in the allergic mice.

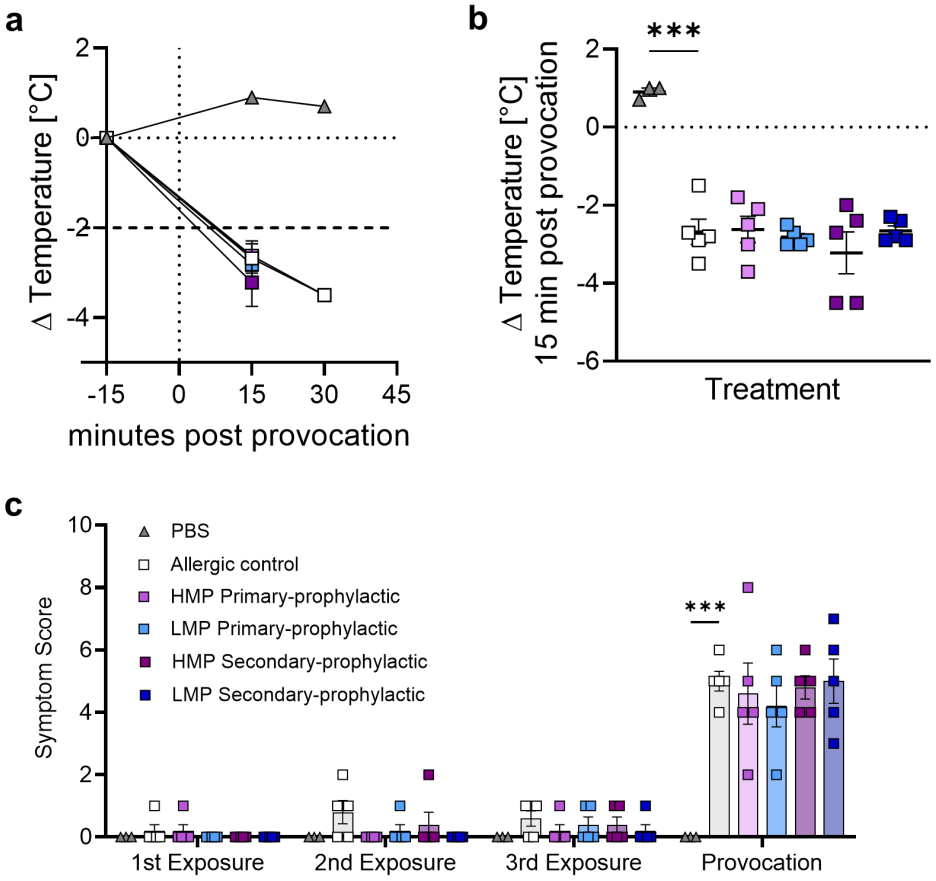

Figure 54: Allergy-related signs after provocation and intervention with HMP or LMP.
(a) Body temperature was measured 15 min before (-15; baseline) and up to 30 min after provocation and (b) is shown for the individual mice 15 min after provocation. (c) The symptom score was examined in a blinded way after each oral exposure as well as after provocation. n = 3-5; ***p < 0.001.

4.5.2 HMP and LMP exert distinct immune-modulatory properties

The effect of HMP or LMP intervention on the allergy-related immune response in peach allergic mice was examined by analysis of PE- or Pru p 3-specific antibody levels in the serum of the mice (Figure 55). Mice of the allergic control showed a significant increase of PE- (Figure 55a) and Pru p 3-sIgE (Figure 55d), that was reduced to baseline values (when compared to the PBS control) by pectin intervention, regardless of the pectin type used and intervention approach. An enhancement of the PE- and Pru p 3-sIgG1 (Figure 55b,e) and IgG2a levels (Figure 55c,f) was observed in all PE-sensitized groups, with no clear effect by the pectin treatment, besides a slight reduction of sIgG1 by HMP primary-prophylactic

intervention. The ratio of PE- and Pru p 3-specific IgG1 to IgG2a was significantly enhanced in the allergic control group compared to the negative control but slightly reduced by all pectin interventions (Figure 55g,h).

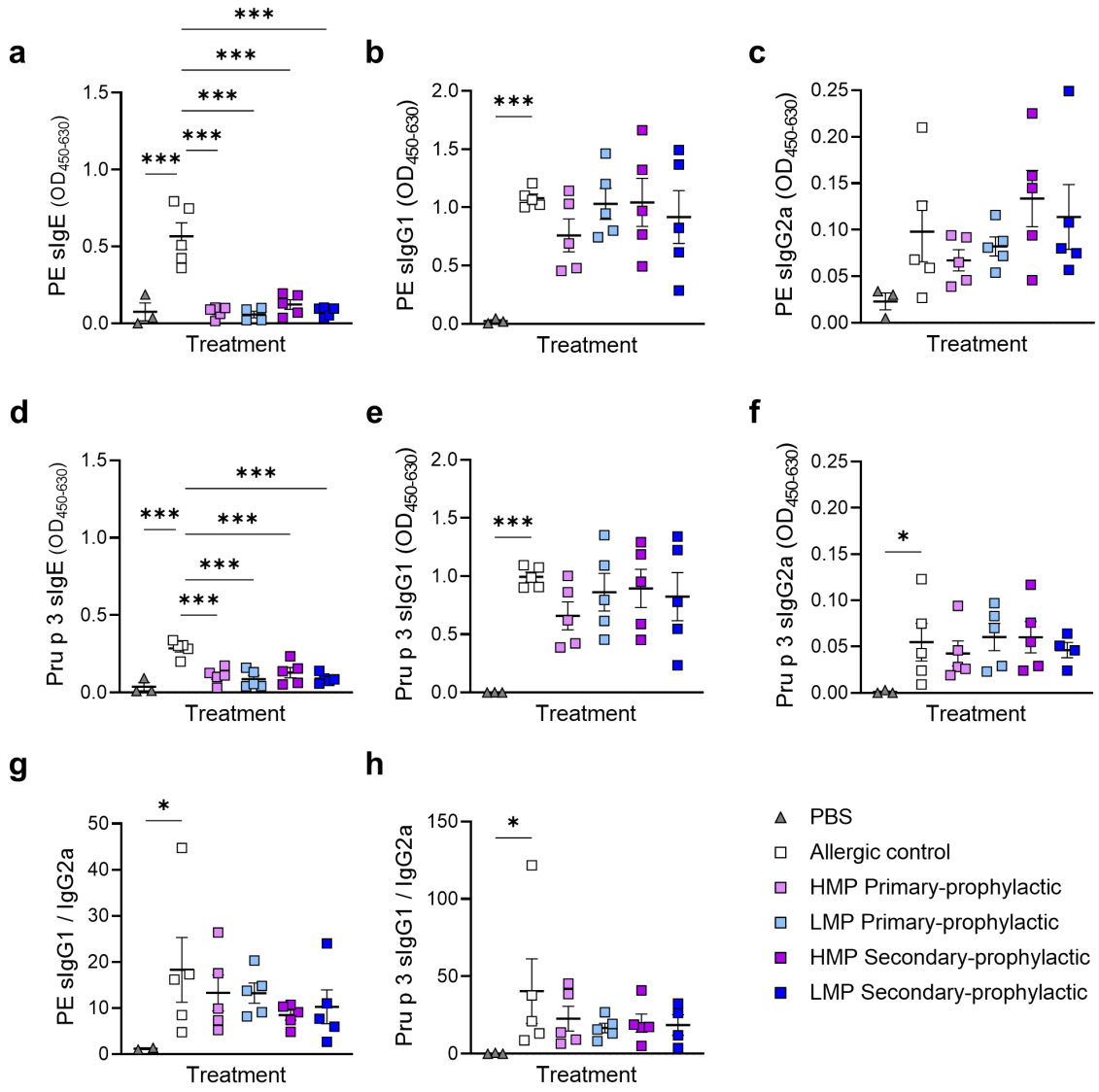

Figure 55: Allergen-specific antibody response in the serum after HMP or LMP intervention. PE- and Pru p 3-specific antibody response was examined in the serum of the mice after provocation. Levels of PE-specific (a) IgE, (b) IgG1 and (c) IgG2a, as well as Pru p 3-specific (d) IgE, (e) IgG1 and (f) IgG2a were analyzed by ELISA. Ratio of (g) PE-specific and (h) Pru p 3-specific IgG1 to IgG2a was determined. n = 3-5; *$p < 0.05$; ***$p < 0.001$.

In addition to specific antibodies, levels of mMCPT-1 and total Igs were measured (Figure 56). mMCPT-1 levels were significantly enhanced in the allergic control mice

compared to the PBS control but substantially decreased by pectin intervention, with no difference between the pectin type or intervention approach (Figure 56a). Furthermore, levels of total IgE were tendentially enhanced in the allergic control group compared to the PBS control without reaching significance (Figure 56b). Interestingly, only HMP primary-prophylactic intervention tended to reduce the levels of total IgE, whereas no effect could be observed in the other pectin intervention groups. Levels of total IgG were significantly enhanced in the allergic control, with only primary-prophylactic LMP slightly reducing these levels (Figure 56c). Total IgA levels were significantly reduced in the allergic mice when compared to the PBS controls, whereas intervention with HMP was able to restore the levels of total IgA in the serum to normal conditions (Figure 56d). The ratio of total IgG to total IgE was enhanced after primary-prophylactic HMP intervention compared to the allergic control (Figure 56e).

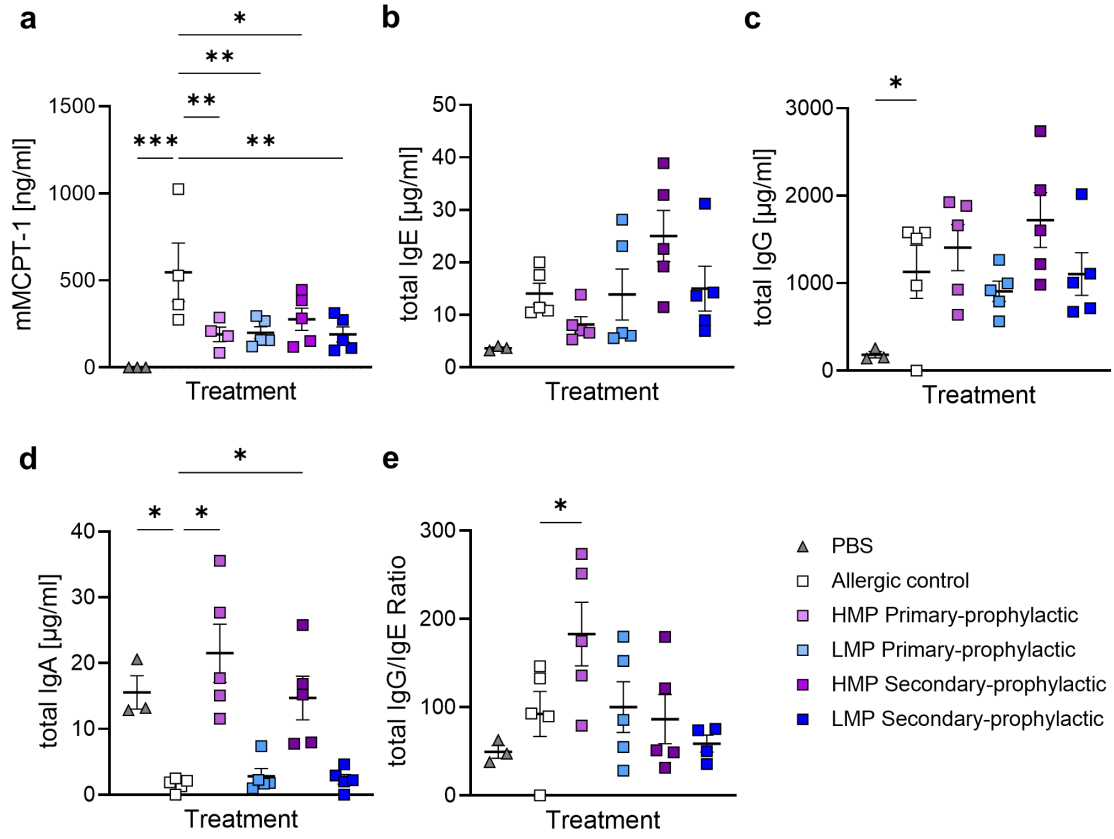

Figure 56: mMCPT-1 levels and total immunoglobulin levels after HMP or LMP intervention. Levels of (a) mMCPT-1, (b) total IgE, (c) total IgG, (d) total IgA and (e) total IgG/IgE ratio were examined in the serum of the mice after provocation. n = 3-5; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Furthermore, cytokine secretion was analyzed in the supernatant of splenocytes or cells of the MLN, re-stimulated with PMI + Ionomycin. In splenocyte supernatant, levels of IL-4 were significantly enhanced with both secondary-prophylactic interventions (Figure 57a). Levels of IL-13 were tendentially enhanced in splenocyte supernatants after both HMP intervention approaches (Figure 57b), whereas levels of IL-5 and IFNγ showed no differences between the groups (Figure 57c,d).

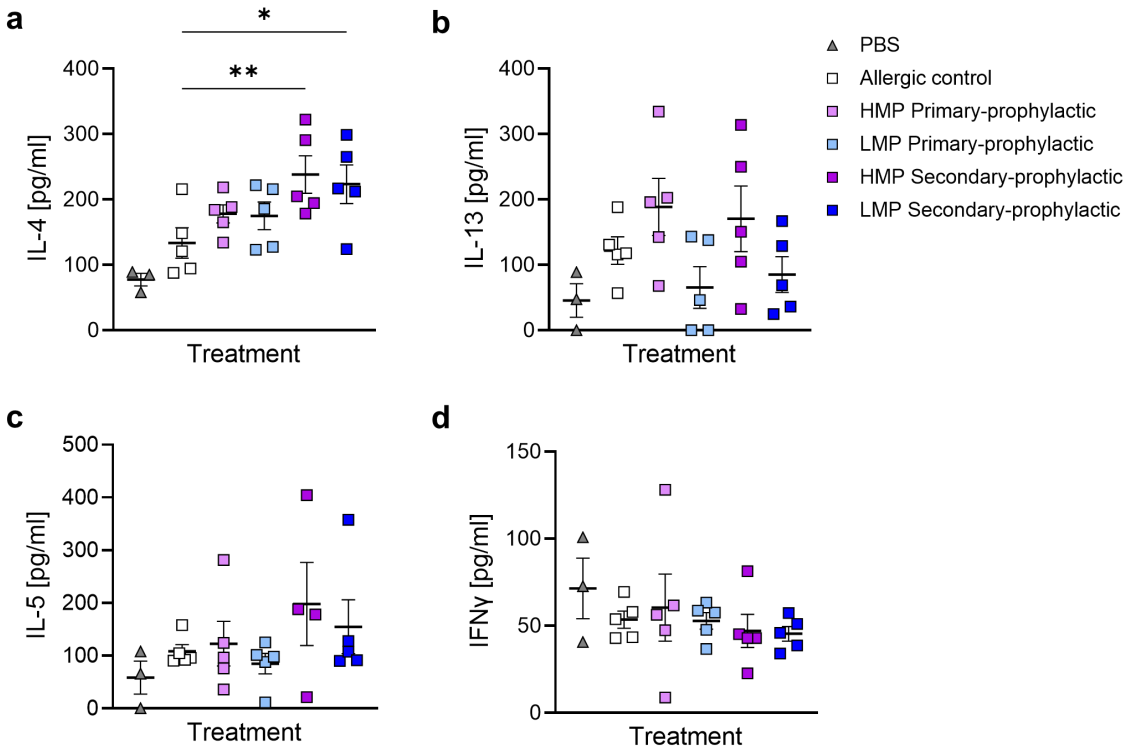

Figure 57: Cytokine secretion in splenocyte supernatant after pectin intervention.
Splenocytes were re-stimulated with PMI + Ionomycin for 48 h followed by measurement of cytokines in the supernatant by ELISA. n = 3-5; *$p < 0.05$; **$p < 0.01$.

Re-stimulation of the cells of the MLN revealed a different effect on the cytokine levels (Figure 58). Levels of IL-4 were tendentially enhanced in the allergic control and both primary-prophylactic interventions compared to the PBS control (Figure 58a). Secondary-prophylactic intervention, especially with HMP significantly decreased these levels. A similar decrease was observed for levels of IL-13, whereas primary-prophylactic intervention with LMP significantly enhanced IL-13 levels compared to the allergic control (Figure 58b). Levels of IL-5 were strongly

enhanced in all PE-immunized groups compared to the PBS control, whereas primary-prophylactic intervention with HMP as well as secondary-prophylactic intervention with LMP significantly reduced these levels to baseline (Figure 58c). Levels of IFNγ were slightly enhanced in the peach allergic control and the primary-prophylactic HMP intervention but strongly increased by primary-prophylactic LMP intervention (Figure 58d).

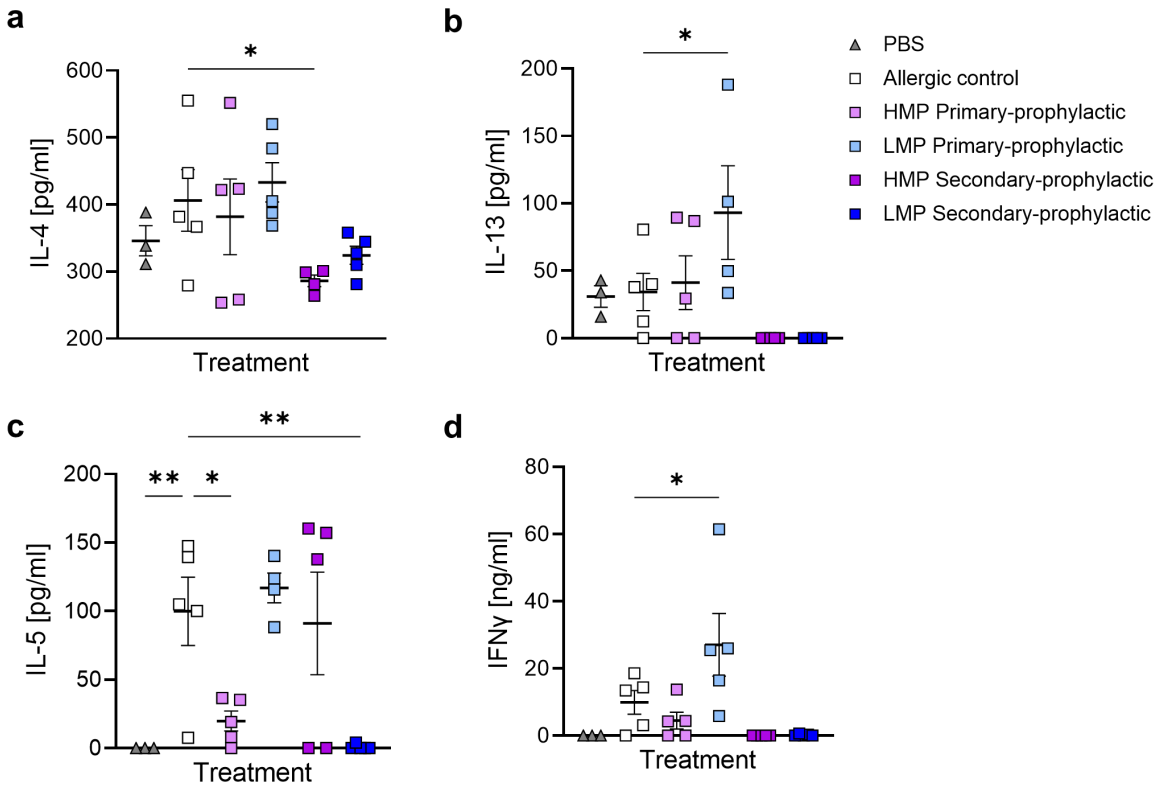

Figure 58: Cytokine secretion in MLN supernatant after pectin intervention.
Cells of the MLNs were re-stimulated with PMI + Ionomycin for 48 h followed by measurement of cytokines in the supernatant by ELISA. n = 3-5; *$p < 0.05$; **$p < 0.01$.

4.5.3 HMP and LMP modulate microbiota composition in allergic mice

In line with the microbiota analysis performed for HMP intervention in allergic mice, the effect of the two structurally different pectins HMP and LMP on microbial composition was examined by 16S rRNA analysis of fecal samples. This analysis was done in collaboration with other departments of the Paul-Ehrlich-Institut. Dr. Csaba Miskey (Division of Medical Biotechnology; Paul-Ehrlich-Institut, Langen,

Germany) performed sequencing of the DNA library and Dr. Oleg Krut (Microbiological Safety Section; Paul-Ehrlich-Institut, Langen, Germany) did the bioinformatic analysis of the results and kindly provided these data.

The results indicated that the microbial richness, calculated as Chao1 index, was significantly decreased 4 days after start of HMP intervention (day -2 for HMP primary-prophylactic, day 16 for HMP secondary-prophylactic) (Figure 59). Interestingly, no effect on microbial richness was observed after LMP intervention. Also, in the peach allergic control as well as in the negative control, the richness was not altered between the different sampling days.

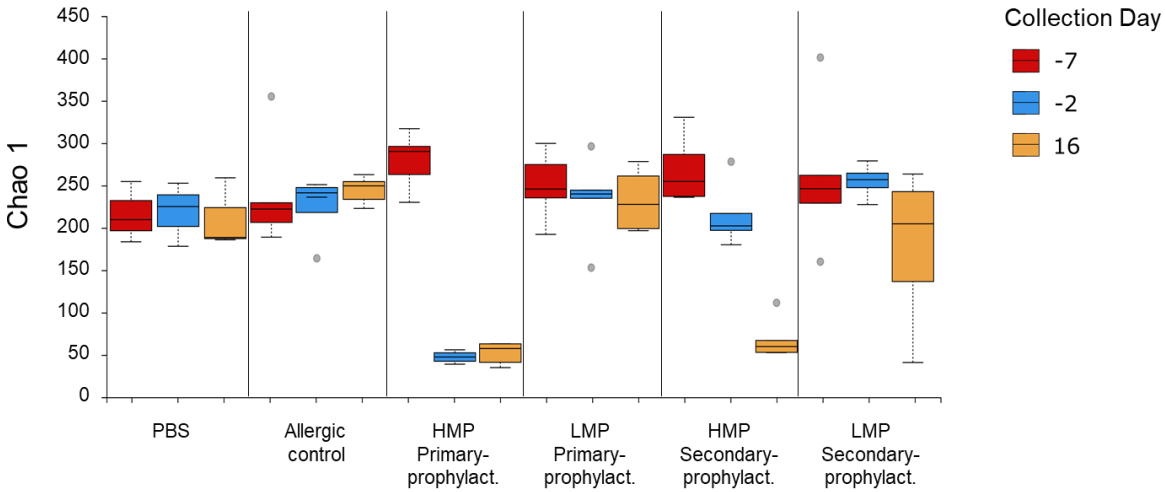

Figure 59: Effect of HMP or LMP intervention on microbial richness.
Microbial content was analyzed in feces samples at different time points. Microbial richness was calculated as Chao1 index. Sequencing of the isolated DNA samples was done by Dr. Csaba Miskey and bioinformatic analysis was performed by Dr. Oleg Krut from the Paul-Ehrlich-Institut, Langen, Germany.

Furthermore, analysis of bacterial composition revealed a strong shift towards *Bacteroides* 4 days after start of the pectin intervention (Figure 60). In contrast to the microbial richness, this effect occurred with both structurally different pectins and both interventions. Whereas *Bacteroides* increased after start of the pectin intervention, *Alistipes* was shown to be decreased in these groups but the levels stayed constant in the allergic and the negative controls. After start of the primary-prophylactic LMP intervention, members of *Lachnospiraceae* increased which was tendentially observed also in the secondary-prophylactic LMP group.

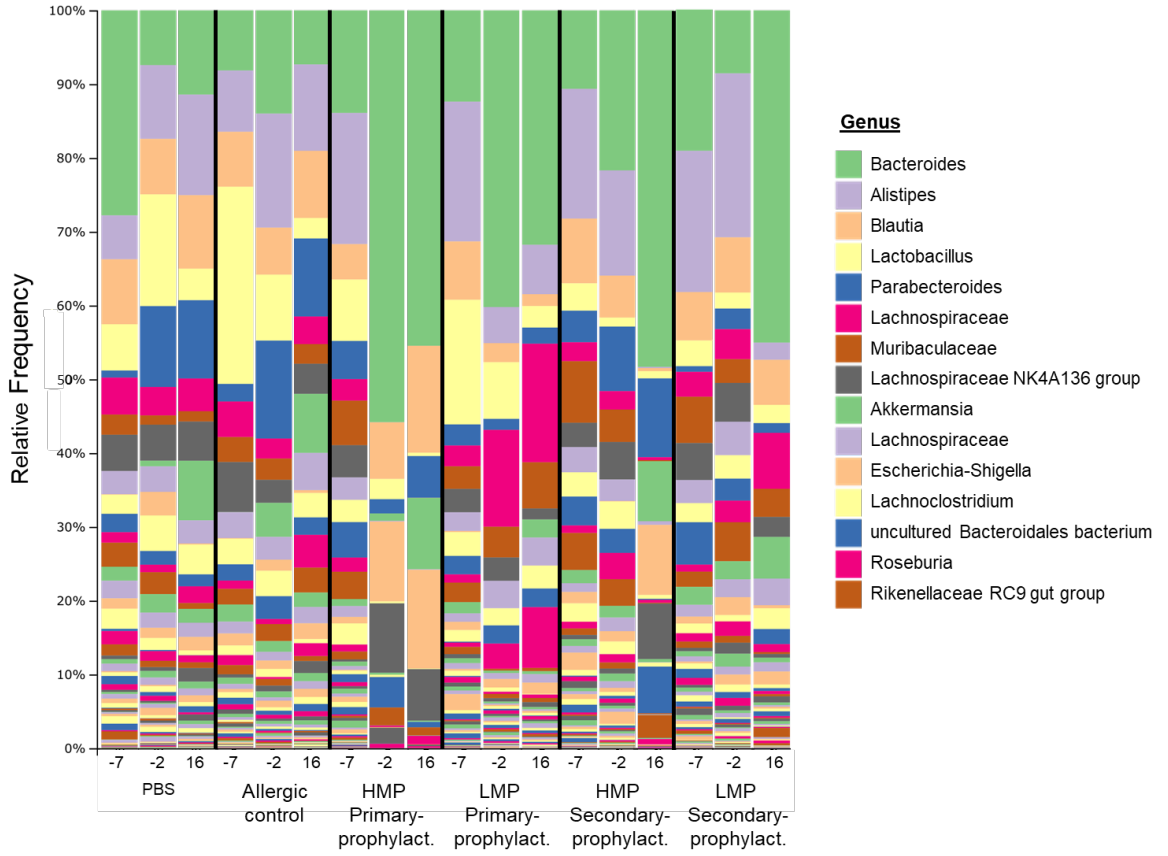

Figure 60: Modulation of gut microbiota composition by HMP or LMP intervention.
Relative frequency of bacterial genera was examined in feces samples of the mice at five different time points by 16S-rRNA analysis. Sequencing of the isolated DNA samples was done by Dr. Csaba Miskey and bioinformatic analysis was performed by Dr. Oleg Krut from the Paul-Ehrlich-Institut, Langen, Germany.

4.5.4 Effects of HMP and LMP intervention on intestine physiology and cellular immune response

Considering the systemic effects observed with both pectins on the humoral immune response, the local effects of pectin intervention in the intestine were evaluated. The length of the small intestine was significantly enlarged by both HMP interventions compared to the allergic control (Figure 61a). Similarly, the large intestine was enlarged after HMP intervention, especially after primary-prophylactic intervention (Figure 61b). Also, the caecum was significantly increased after both intervention approaches with HMP and secondary-prophylactic intervention with LMP (Figure 61c).

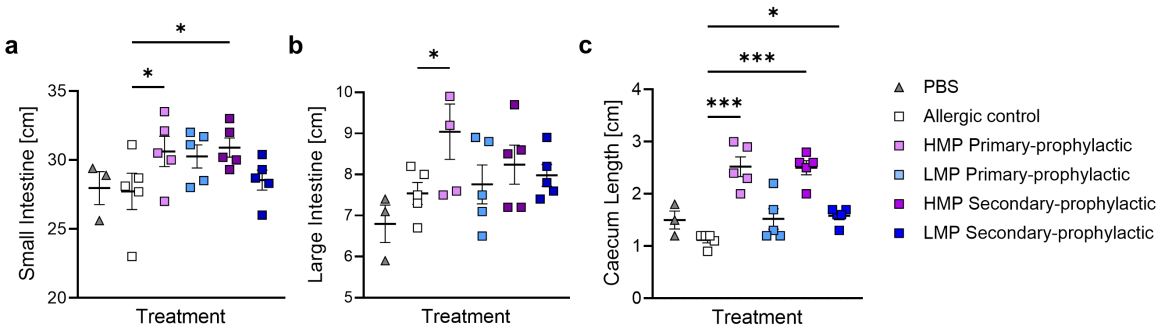

Figure 61: Length of small and large intestine and caecum after HMP or LMP intervention. Length of the (a) small intestine, (b) large intestine and (c) caecum was evaluated after provocation and euthanasia of the mice. n = 3-5; *$p < 0.05$; ***$p < 0.001$.

Measurement of mMCPT-1 and total Ig levels in the supernatant of small intestine homogenates showed a significant increase of mMCPT-1 levels in the allergic control compared to the PBS group. These levels were strongly decreased in both HMP intervention groups but not with LMP (Figure 62a). Levels of total IgE were significantly enhanced in the allergic control compared to the PBS control but were reduced by all pectin interventions (Figure 62b). However, both secondary-prophylactic interventions induced stronger decrease of total IgE compared to the primary-prophylactic approaches. Similarly, levels of total IgG were enhanced in the mice of the allergic control but significantly decreased after secondary-prophylactic intervention using either HMP or LMP (Figure 62c). The levels of total IgA in the homogenates showed no differences between the six treatment groups (Figure 62d).

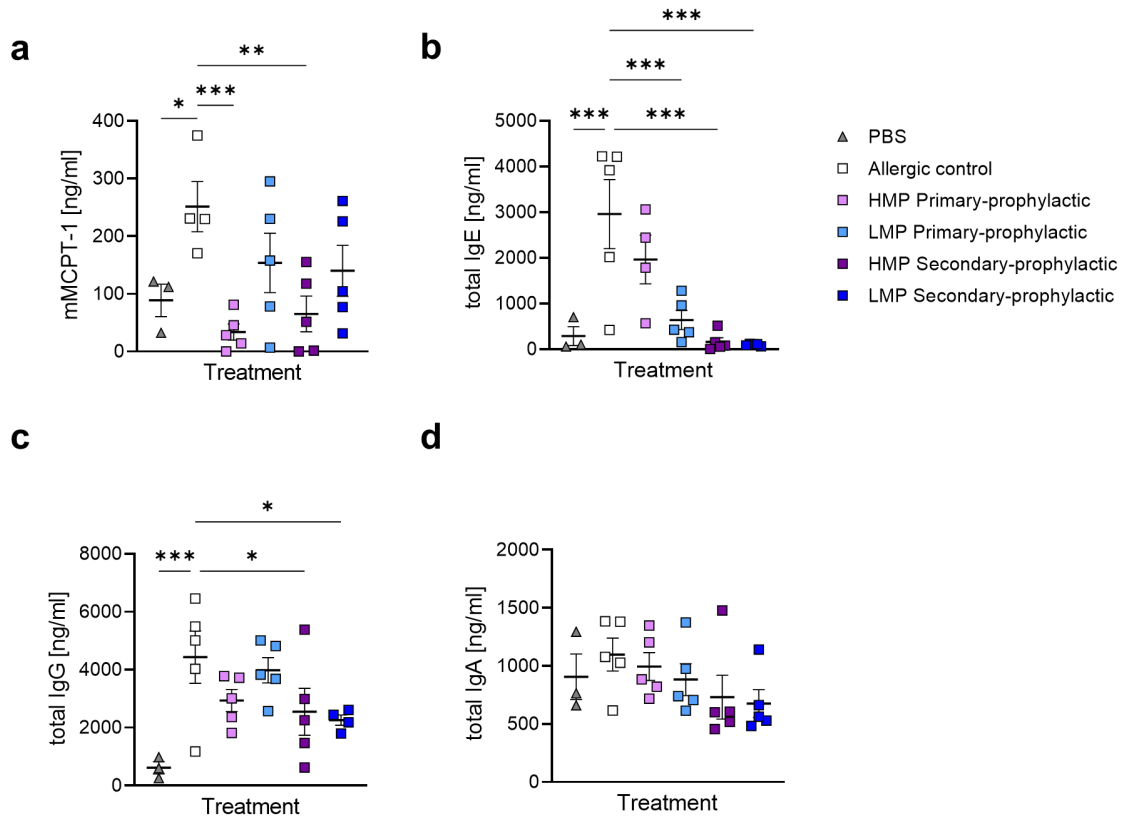

Figure 62: mMCPT-1 and total Ig levels in the homogenates after HMP or LMP intervention. Levels of (a) mMCPT-1, (b) total IgE, (c) total IgG and (d) total IgA were analyzed in the supernatant of intestinal homogenates after provocation via ELISA. n = 3-5; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Infiltrating immune cells were analyzed in the LP of small and large intestine by flow cytometry (Figure 63). Results showed variation in the frequency of mast cells especially in the small intestine. There, mast cell frequency was tendentially increased in the allergic mice, but significantly decreased by secondary-prophylactic pectin intervention (Figure 63a). In the large intestine, only secondary-prophylactic HMP intervention significantly reduced mast cell frequencies compared to the allergic control. Eosinophils and macrophages were strongly decreased in the large intestine by all pectin interventions compared to the allergic control. In the small intestine, only a slight reduction of eosinophils was observed by both primary-prophylactic interventions, whereas macrophages were significantly reduced by primary-prophylactic HMP intervention (Figure 63b,c). Additionally, the amount of cDCs was not altered in the small intestine, whereas in the large intestine both HMP interventions led to significantly decreased levels of cDCs (Figure 63d).

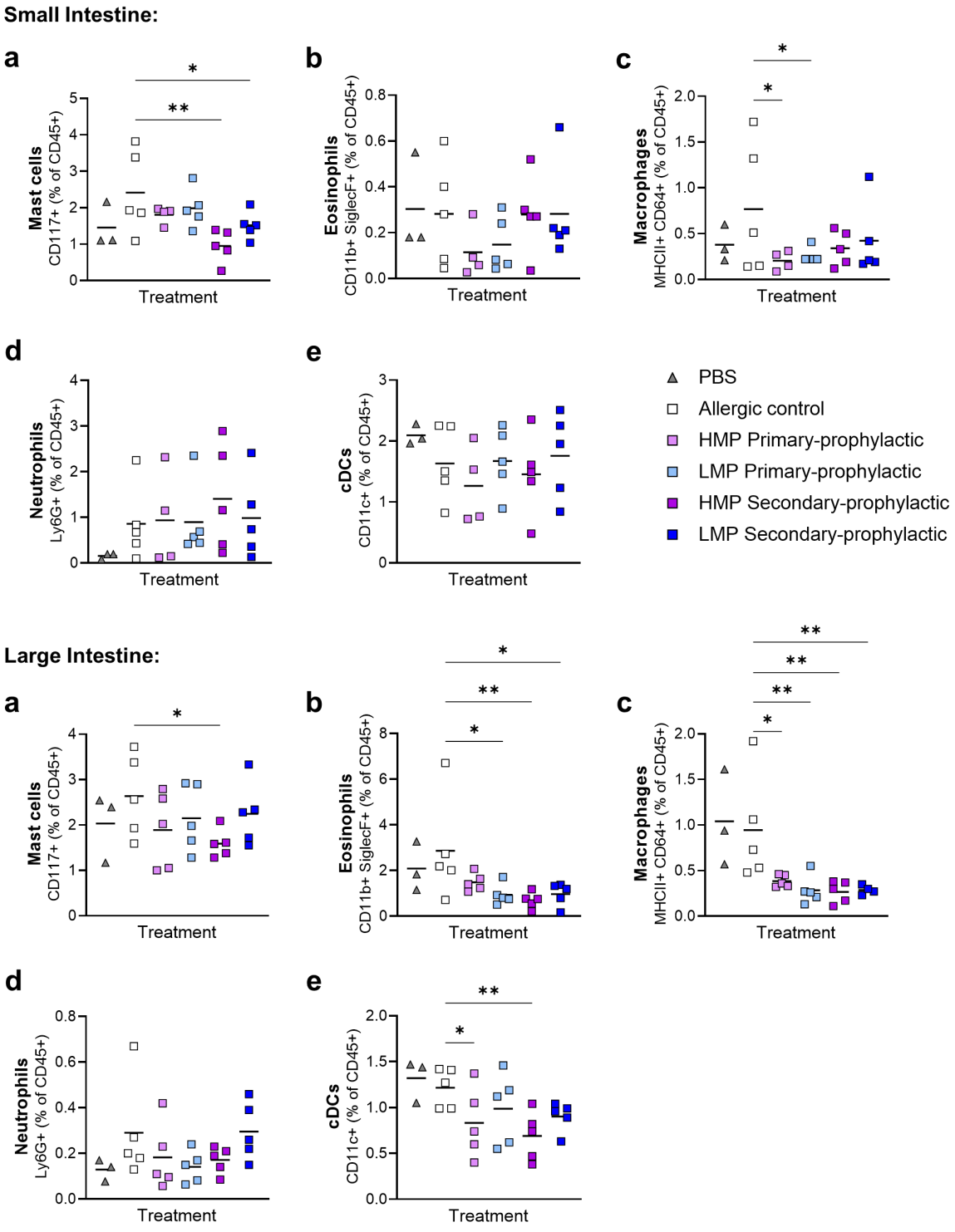

Figure 63: Analysis of infiltrating granulocytes in the lamina propria of small and large intestine.

Small and large intestine were enzymatically treated and isolated LP immune cells were analyzed via FACS. (a) Mast cells, (b) eosinophils, (c) macrophages, (d) neutrophils and (e) conventional dendritic cells (cDCs). n = 3-5; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

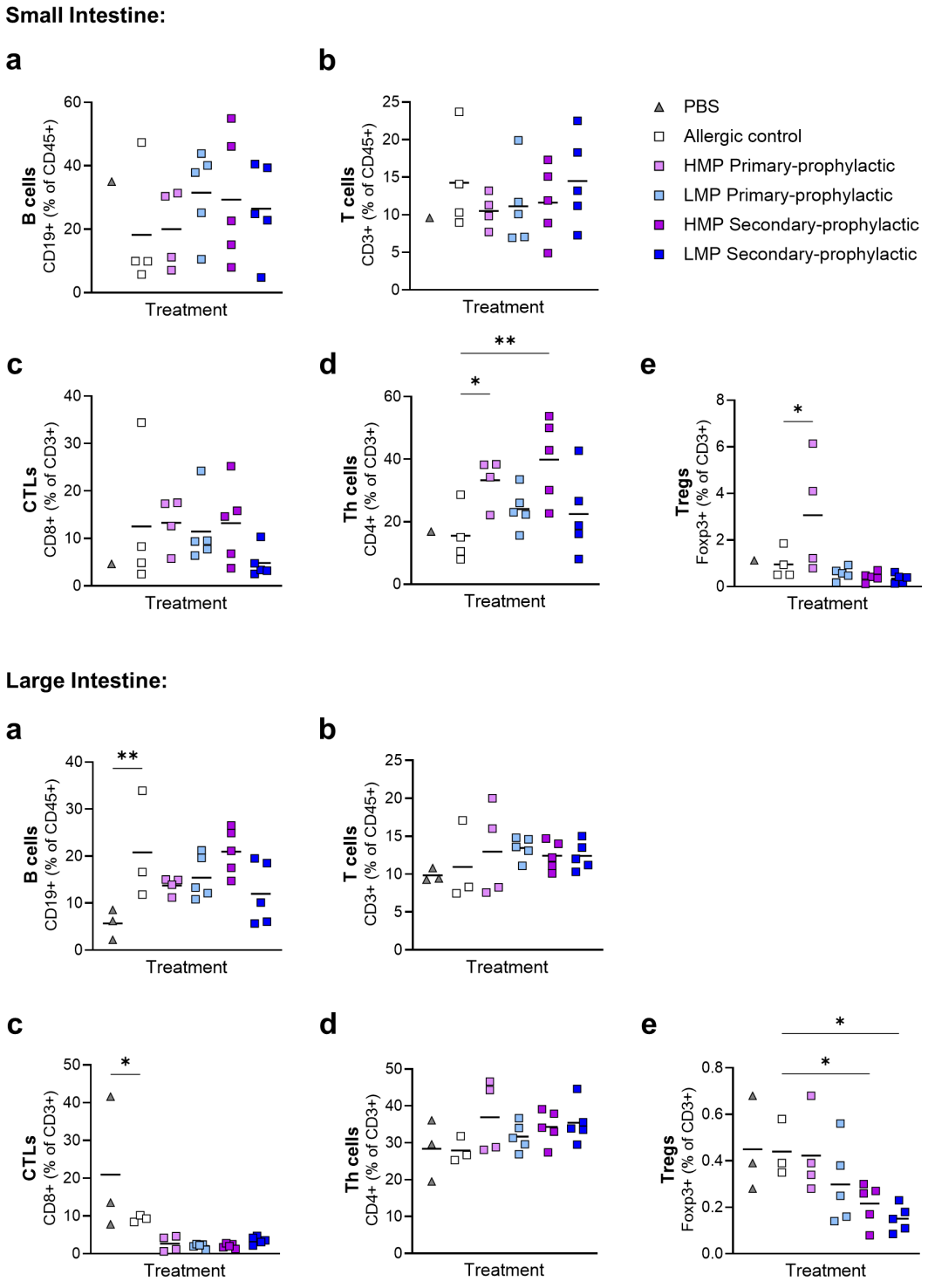

Figure 64: Analysis of infiltrating T and B cells in the lamina propria of small and large intestine.

Isolated infiltrating cells of the LP of small and large intestine were analyzed by FACS. (a) B cells, (b) T cells, (c) cytotoxic T cells (CTLs), (d) T helper cells (Th cells) and (e) regulatory T cells (Tregs). n = 1-5; *$p < 0.05$; **$p < 0.01$.

Further characterization of B- and T-cell subsets in the small and large intestine revealed increased levels of B cells in the allergic control which were only observed in the large intestine (Figure 64a). In comparison, T cells seemed not to be affected by pectin intervention or PE-immunization (Figure 64b). In the large intestine, the frequency of CTLs was strongly decreased in all PE-immunized groups (Figure 64c), whereas Th cells were enhanced in the small intestine by both HMP intervention approaches (Figure 64d). Furthermore, levels of Tregs were significantly enhanced in the small intestine by primary-prophylactic intervention with HMP. In contrast, in the large intestine both secondary-prophylactic intervention approaches led to decreased levels of Tregs compared to the allergic control (Figure 64e).

The data obtained showed that intervention with HMP but also with LMP exerted immune-modulatory effects in a mouse model of FA. However, the structural differences of the pectins as well as the two intervention approaches tested showed distinct modulatory effects, indicating a role of the pectin structure as well as the time and duration of the intervention on the beneficial, modulatory effects.

5 Discussion

5.1 Risk assessment of pectin for nsLTP allergic patients

Pectins play an important role in food industry and are frequently used as gelling or thickening agents and stabilizers (Vanitha and Khan 2020). Besides, different health benefits, including maintenance of blood glucose and lowering cholesterol levels, and also modulation of immune responses and suppression of allergic asthma have been reported after pectin consumption (EFSA Panel on Dietetic Products, Nutrition and Allergies 2010; Trompette et al. 2014). In line with this, the present study aimed to evaluate the effect of pectin on FA, particularly peach allergy. Since the collaborative part of the study aimed to determine the effect of pectin intervention on peach allergic patients, it was important to evaluate the safety of the pectins in regard to the presence of potentially cross-reactive allergens. As the commercial pectins used in this study were extracted from apple or citrus, which are both described to contain allergens of the nsLTP family, they might pose a risk for nsLTP allergic patients due to potential remaining of active allergens during the commercial production. As a consequence of the high similarity between different nsLTPs, it was reported that patients show allergic reactions against a variety of nsLTPs from different plant species, with symptoms ranging from mild to severe anaphylactic reactions after contact with the allergen (Asero et al. 2002). Considering this high cross-reactivity of nsLTP-specific IgEs, especially from *Rosaceae* plants like peach or apple, the potential risk of pectin consumption for peach allergic patients needed to be evaluated, especially when taking into account the high amount of pectin supplemented for the dietary intervention (Skypala et al. 2021a).

The average daily intake of pectin from a regular or even high fiber human diet has been defined as 2 - 38 mg/kg body weight, resulting in an estimated maximum consumption of 2.6 g pectin assuming an average body weight of 70 kg (Mortensen et al. 2017; Steigerwald et al. 2021). However, the patients included in the dietary intervention study consume higher amounts of pectin (10 g per serving/2 times per day), as this amount was proposed to achieve the stated health claims. Due to the high pectin intake of the nsLTP allergic patients, the characterisation of residual

allergen content in the pectins is of particular importance (EFSA Panel on Dietetic Products, Nutrition and Allergies 2010).

To assess the risk for peach allergics, the nsLTP thresholds that might elicit allergic reactions were estimated based on dose-dependent food (peach) challenge experiments with known Pru p 3 content (unpublished data from collaboration partners at IBIMA, personal communication of Prof. Maria Torres). To that end, different methods were established to increase sensitivity and to detect potential residual nsLTP concentrations in the two used pectins. However, presence of the pectins interfered with the detection methods and hampered allergen detection. This might be due to matrix effects, as previously reported pectin-based matrix effects were shown to interfere with allergenic proteins and might also hamper protein detection (Polovic et al. 2007). To overcome this interference, pectin samples were incubated with Fructozym®, a pectinase (endo-polygalacturonase) that catalyses the random hydrolysis of α-(1-4)-linked-D-galactosiduronic linkages within the HG units (Mill and Tuttobello 1961; Latarullo et al. 2016).

The pectinase treatment reduced the interference of HMP as both spiked nPru p 3 concentrations could be detected by immunoblot in HMP but not LMP samples. Unfortunately, also in HMP the pectin matrix still interfered with the detection method, seen by the decreased AUC in pectin samples when compared to the nsLTP without pectin. The differences observed in protein detectability between the two pectins might occur due to several structural differences, such as DE, distribution of side chains or GalA content. It was reported that the DE affects the gelling characteristics as well as the formation of pectin-protein complexes, suggesting a higher interaction between protein and LMP (Yavuz-Düzgün et al. 2020; Gawkowska et al. 2018; Steigerwald et al. 2021). Thus, protein detection might be more difficult in LMP compared to HMP. Surprisingly, enzymatic treatment increased the protein detectability especially in HMP. This was not expected, as the used endo-polygalacturonase was reported to prefer non-esterified substrate, showing decreasing activity with an increasing DE (Babbar et al. 2016). However, pectins with similar DE might still show different types, numbers and distributions of side chains, that might interact with the protein or hamper enzymatic digestion. Besides the classification as HMP or LMP, the distribution of the methyl-esters was

reported to play an important role for enzymatic digestion (Daas et al. 1999). The so-called “degree of blockiness” (DB) quantitatively describes the amount of non-esterified mono-, di- and trigalacturonic acids released by enzymatic treatment with endo-polygalacturonase relative to the total amount of non-esterified GalA residues (Daas et al. 1999; Jermendi et al. 2022). However, pectins with similar DE and DB might still differ in their methyl-esterification patterns. This influences their digestibility, as endo-polygalacturonases were shown to need at least four consecutive non-esterified GalA residues for cleavage (Guillotin et al. 2005; Jermendi et al. 2022).

To enhance the protein detection and reduce the interference by the pectin matrix, precipitation of protein or pectin from the solution was performed (Figure 14). Both precipitation approaches enabled in both pectins the detection of the spiked nPru p 3 in the supernatant, at an allergen concentration representing the threshold for anaphylactic reactions. In addition, mediator release assay using huRBL-30/25 cells indicated no interference of the pectin matrix as both spiked protein concentrations were detectable in both enzymatically treated pectins.

The results of the pectin characterisation regarding residual nsLTPs, suggest that detection sensitivity of different methods differs depending on the structure of the pectins used. Importantly, spiked nsLTP, at a concentration representing the threshold that might elicit anaphylaxis in allergics was detectable in both pectins, whereas no nsLTP protein was found in any of the unspiked pectin samples. It can be concluded that the potential residual nsLTP content in both commercial pectins is with high probability below the nsLTP threshold eliciting severe anaphylactic reactions, posing no risk for such a reaction to patients consuming up to 10 g of pectin in a serving.

5.2 Immune-modulatory effect of pectin on naïve mice

To evaluate any adverse effects and to discern whether structurally different types of pectin exert distinct immune-modulatory effects, HMP or LMP were supplemented in two different amounts to naïve mice. Furthermore, the effects of a pectin-enriched diet were compared in three different inbred mouse strains.

Although it was reported, that pectin supplementation might reduce body weight in obese mice (Drew et al. 2018), HMP supplementation did not affect body weight gain or food intake of the healthy mice during the course of the experiment in the three mouse strains that were examined. In contrast, LMP supplementation led to significantly increased body weight in BALB/c mice in comparison to the control group, that could be correlated to enhanced food intake. It was reported, that BALB/c mice tend to gain more body weight in comparison to C57BL/6 mice under high-fat diet due to immunometabolic differences (Jovicic et al. 2015). In relation to the average food intake (estimated for CBA/J mice), the daily pectin consumption was estimated as 150 mg (5% pectin) or 450 mg (15% pectin) per mouse, which can be assumed as a high-pectin diet. However, histological analysis indicated no disease-related symptoms or inflammatory processes even after high amounts of pectin supplementation (Figure 26).

Pascale et al. (2022) found that immune-modulatory effects promoted by pectin can be indirectly induced by modulation of the gut microbiota composition. The authors reported that MW and DE of pectins modulate their fermentability and the impact on the microbiota composition and immune response. In line with this, the present results supported the assumption that especially supplementation of HMP modulated the gut microbiota, characterised by a strong shift towards *Bacteroides*, while only a weak effect was observed after supplementation of LMP. This might be due to differences in the fermentability of the two examined pectins, as unpublished data of collaboration partners at the Karlsruhe Institute of Technology (KIT) showed a faster fermentation of LMP compared to HMP *in vitro* (personal communication by Prof. Mirko Bunzel, Department of Food Chemistry and Phytochemistry, Karlsruhe Institute of Technology, Karlsruhe, Germany). Among others, *Bacteroides* species are reported to produce mediators, including SCFAs that can induce certain immune-responses by interaction with e.g. TLRs and GPCRs (Rios-Covian et al. 2013). In this way, bacterial strains can promote pro- as well as anti-inflammatory immune responses and affect frequencies and differentiation of immune cells (Liu et al. 2023).

Furthermore, a strong decrease of alpha diversity after supplementation of HMP was observed in all mouse strains tested. This result is in line with results reported

from *in vitro* fermentation experiments with structurally different pectins or from mouse models (Larsen et al. 2019; Nakamura et al. 2023). However, microbial diversity also decreased in the control groups of C57BL/6 and BALB/c mice, which was not observed in CBA/J mice. Studies could show that the insoluble fiber cellulose (contained in the control diet) is barely fermented by the mammalian gut microbiota but might still induce changes in the composition of gut bacteria, such as promotion of the growth of *Akkermansia muciniphila* (Nagy-Szakal et al. 2013; Kim et al. 2020). However, the underlying mechanism is still topic of investigation. Nevertheless, cellulose-containing control diet was fed to the mice for two weeks before start of the experiment (d0), leading to the conclusion that the potential effects induced by cellulose are slower and not comparable to the fast changes in microbiota composition induced by pectin. In addition, this decrease of diversity in the control group was not observed in all investigated mouse strains.

Moreover, the substantial increase of the relative abundance of *Bacteroides* after HMP supplementation observed in the three mouse strains might explain the significant drop of alpha diversity in these groups. It is described, that members of the *Bacteroides* genus express a high variety of pectin degrading enzymes and therefore can quickly respond to polysaccharides in the environment (Ndeh and Gilbert 2018; Martens et al. 2011; Chung et al. 2017). *Bacteroides* are therefore reported as primary pectin degraders among the intestinal bacteria (Luis et al. 2018).

Interestingly, supplementation of either HMP or LMP showed clear differences regarding not only the modulation of gut microbiota composition but also the humoral immune response. In all mouse strains, enhanced levels of total serum IgA and IgG were found, especially after HMP supplementation (Figure 23). Additionally, the ratio of total IgG to total IgE was significantly increased after 15% HMP supplementation. It has been reported, that modulations of the gut microbiota composition could result in increased serum IgA and IgG levels but not IgE levels, due to enhanced B cell differentiation and antibody production promoted by SCFAs (Wilmore et al. 2018; Zeng et al. 2016; Ishikawa and Nanjo 2009). The enhanced levels of serum IgA may be associated with beneficial effects on FAs, due to potential IgE-blocking capacities and inhibition of IgE-mediated mast cell activation

by specific IgA (Shamji et al. 2021; El Ansari et al. 2022). Thus, supplementation of HMP might have protective effects and lower the risk for development and manifestation of FAs.

A high IgG/IgE ratio reflects an immune deviation from Th2 (IgE) to a Th1 (IgG), at which specific IgG antibodies likely can act as “blocking” antibodies that compete with IgE and inhibit activation of basophils and mast cells by binding to the inhibitory FcγRIIb (Eckl-Dorna et al. 2018; Kanagaratham et al. 2020; Burton et al. 2018). This is of particular interest, considering that the focus of this study was to evaluate the immune-modulatory effects of pectin on allergic diseases. Notably, despite the different immunological background of the mouse strains (e.g. C57BL/6 mice preferentially develop Th1 immune response and BALB/c mice commonly develop a Th2-prone immune response (Jovicic et al. 2015; Watanabe et al. 2004)) HMP supplementation promoted a balance towards Th1 over Th2 in all mouse strains investigated

Additionally, local effects, such as enlarged intestine sections were detected after pectin supplementation (Figure 25). It was reported, that feeding of a pectin-enriched diet to rats resulted in enlarged intestine sections due to mucosal hyperplasia (Chun et al. 1989; Adam et al. 2015). In this study, supplementation of HMP led to enlarged caecum in all mouse strains and increased large intestine in CBA/J mice. In comparison, only in BALB/c mice supplementation of 15% LMP was also capable to increase the length of the caecum. These differences are likely depending on the structural characteristics of pectin as well as different fermentation processes, as LMPs are fermented more efficiently by the microbiota in the ileum, whereas HMP is mainly fermented in the proximal colon (Tian et al. 2017). However, the intestinal microbiota of the different mouse strains that was present before start of the experiment might influence the pectin fermentation and thereby the length of intestinal sections.

In addition to indirect immune-modulation by gut microbiota, it is reported that pectins can directly interact with immune cells, and this effect is mainly observed in the small intestine (Beukema et al. 2020; Agace and McCoy 2017). Supplementation of 15% HMP significantly influenced the abundance of immune cells in the LP of the small intestine when compared to LMP (Figure 27). A reduced

frequency of eosinophils and mast cells, as well as macrophages and DCs was observed when 15% HMP was supplemented. These cells play a role in inflammatory immune responses as well as antigen presentation and pectin-induced reduction might indicate a protective role of HMP in regard of inflammatory processes and allergy. In addition, frequencies of B cells and Th cells were increased by HMP, whereas LMP led to decreased frequencies of T cells. These results are in line with previously observed structure-dependent effects of pectin on T cell immunity (Beukema et al. 2022). It remains elusive whether the local cellular immune response is mediated by direct interaction with pectin or indirectly via gut microbiota modulation. However, studies could show enhanced numbers of CD4- and CD8-positive cells in the LP of the intestine after feeding of prebiotic bacteria, supporting an immune-modulatory role of the gut microbiota (Moreno LeBlanc et al. 2008).

In summary, the results obtained in these experiments suggest that the immune-modulatory capacity of pectins correlates with their chemical structure and fermentability. The data revealed a differential effect of two pectins with different DE in naïve mice. In particular, pectin with high methoxyl content (HMP) was fermented more slowly and altered the gastrointestinal microbiota composition substantially, e.g. towards increased frequencies of *Bacteroides*. This changes in the gut microbiota by HMP might be responsible for the elicited immune-modulatory effects by HMP, inducing strong IgA and IgG/IgE responses and influencing local cellular immune responses. In comparison, LMP induced only a slight shift in microbiota composition into the direction of *Bacteroides* and the observed immune-modulatory effects were weaker compared to HMP. Together with members of *Clostridia* family originated from human, *Bacteroides* have been reported to potentially inhibit FA by mediator production and modulation of especially T cell immune response (Abdel-Gadir et al. 2019; Bunyavanich and Berin 2019). However, the correlation between fermentability, the shift in the gut microbiota, as well as the immune-modulatory properties, and the suggested contribution to the reduction of the inflammatory allergic immune response needs to be further investigated.

5.3 PE induced allergy-related signs in a peach allergy mouse model

Mouse models are commonly used to mimic and study mechanistic effects of FA (Wang et al. 2022; Birmingham et al. 2005). A mouse model of peach allergy was established to this end. CBA/J mice were used due to prior observations, suggesting a strong Th2-biased immune response upon sensitization with nsLTPs (data not shown). As shown in this study, a combination of i.p. sensitization and provocation with oral allergen exposure by i.g. administration, resulted in increased levels of sIgE, development of allergy-related clinical signs as well as local immune responses in the gut.

In comparison to a previously reported Pru p 3-allergy mouse model by Rodriguez *et al.*, PE instead of purified allergen Pru p 3 + LPS was used for sensitization and provocation in this study (Rodriguez et al. 2017). This decision was made because the model reported by Rodriguez et al. uses a single allergen and does not induce local inflammatory reactions in the intestinal tract. Preliminary own experiments using nPru p 3 as sensitization agent showed low immunogenicity and no production of sIgE as well as total antibodies (Steigerwald et al. 2023). This effect might occur, as several proteins and allergens are present in the PE, leading to potentially higher immunogenicity and mimics a more natural situation as allergics consume not only Pru p 3 but a whole peach, containing a broad variety of proteins. Mouse serum showed IgG-reactivity against proteins in the PE, recognized mainly as Pru p 3 but also additional proteins in the extract (Figure 34). Based on the MW of the detected reactive proteins, one might speculate that the peach proteins Pru p 7 or Pru p 1 could be included in the extract. These were reported to induce severe symptoms in allergic individuals (Klingebiel et al. 2019; Jin et al. 2021). It should also be considered that the natural ligand of Pru p 3 was reported to enhance allergenicity due to adjuvant like effects, whereas the ligand might not be present when purified allergen is used (Gonzalez-Klein et al. 2021). It was shown that cleavage of the ligands lipid tail inside of epithelial cells induces release of a sphingosine-1-phosphate analogue, which plays an important role in the pathogenesis of allergic diseases as chemoattractant for monocytes (Fuerst et al. 2014; Wedman et al. 2018; Gonzalez-Klein et al. 2021). Moreover, the lipid-ligand of Pru p 3 was shown to be involved in the activation of DCs and CD1-mediated

activation of invariant natural killer T-cells (iNKTs) and thereby might enhance the induction of sIgE (Tordesillas et al. 2017a). Furthermore, adjuvant-like effects of fats, as well as water-soluble matrix components of the extract, such as carbohydrates or proteins can be speculated.

The peach allergy mouse model established in this study revealed a significant increase of Pru p 3- as well as PE-specific antibodies in the serum of the mice, including IgE, IgG1 and IgG2a (Figure 33). The increase of sIgG1 observed in comparison to sIgG2a indicates a mainly Th2-driven immune response (Hochreiter et al. 2003). Additionally, enhanced levels of the Th2 cytokines IL-4 and IL-13 but not the Th1 cytokine IFNγ measured in supernatant of re-stimulated splenocytes of PE-treated mice, suggest a Th2-biased immune reaction (Chinthrajah et al. 2016). Furthermore, strongly enhanced levels of total IgE and total IgG in the serum and the intestinal homogenates of peach allergic mice were detected. In comparison, levels of total IgA were decreased in the serum of the allergic mice, which is in line with studies showing a protective role of IgA antibodies against the development of allergic reactions due to potential inhibition of IgE-induced mast cell degranulation or cytokine production (Fagerås et al. 2011; El Ansari et al. 2022).

In the mouse model established in this study, peach allergic mice developed systemic but also local immune reactions in the intestinal tract. These were mainly characterized by enhanced cell infiltration and Ig production. In the small intestine LP, increased frequency of $CD4^+$ Th cells was observed. This correlated with enhanced cDCs, that might induce activation of naïve $CD4^+$ cells and differentiation into Th2 cells (Humeniuk et al. 2017). Surprisingly, enhanced levels of $CD8^+$ T cells (CTLs) and Tregs could be seen in the LP of peach allergic mice as well. CTLs are commonly not involved in the elicitation of IgE-mediated FA, whereas Tregs ($CD3^+CD25^+Foxp3^+$) suppress sensitization and effector phase in FA by secretion of IL-10 (Palomares 2013). However, it was reported, that also $CD8^+$ CTLs might be involved in the Th2 response to type I FAs, as they might get activated by recognition of food antigens (Yu et al. 2019). Different subtypes of Tregs can be distinguished besides their expression of Foxp3 and CD25, which was not subject of this study. Therefore, the characteristics of the Tregs detected in this project are unknown and it remains elusive whether the cells are present in a resting, activated or functionally

exhausted state, determining their ability to sufficiently suppress allergic inflammation or even promote FA (Yang et al. 2017; Xin et al. 2018; Noval Rivas et al. 2015).

The frequency of mast cells in the LP of peach allergic mice was not altered compared to the controls. Nevertheless, mMCPT-1, a mast cell activation marker was clearly increased in the serum as well as in the intestinal homogenates. This finding suggests mucosal mast cell activation upon sIgE cross-linkage (Ahrens et al. 2012; Brandt et al. 2003). Also, no effects on the frequency of eosinophils were observed, which correlates with the levels of IL-5 measured. IL-5 is a pro-inflammatory cytokine that promotes activation, proliferation and maturation of eosinophils (Pelaia et al. 2019). In line with this observation, it was reported that eosinophil-associated inflammation is less apparent in IgE-mediated FA (Schoos et al. 2020; Simon et al. 2016). However, increased numbers of neutrophils indicated the development of an inflammatory immune response in the mice immunized with PE. This effect was not observed in the control animals (Polak et al. 2019).

5.4 Dietary pectin intervention in peach allergic mice

The data obtained in the thesis showed, that supplementation of the dietary fiber pectin was capable to modulate the gut microbiota composition as well as the immune-response in mice, depending on their structural characteristics. In addition, other studies suggested a suppressive role of pectin on allergy manifestation by inhibition of inflammatory immune responses and promotion of Treg generation (Trompette et al. 2014). However, the role of pectin supplementation in manifestation and severity of FAs as well as the role of the pectin structure was still unclear. Therefore, the effect of different pectins on gut microbiota composition, local and systemic immune reaction in peach allergic mice was evaluated in this study.

Dietary pectin intervention was not sufficient to prevent the body temperature drop of the mice after provocation, and only barely reduced the development of clinical signs (Figure 42, Figure 54). Notably, the allergic reaction was provoked by i.p. injection of a defined allergen dosage, inducing an immediate systemic reaction in

the mice. It might be speculated that in contrast to one defined dosage to induce clinical signs, administration of increasing allergen dosages might reveal an increased allergen tolerance in the pectin treated mice reflected by induction of clinical signs at higher allergen concentrations. Such effects were observed in the dietary intervention study for some peach allergic patients, indicating that both pectins (HMP and LMP) were able to increase the tolerated dosage of peach allergens in around one third of the patients (unpublished data, personal communication of Prof. Dr. Maria Torres, IBIMA, Malaga, Spain).

In contrast to the clinical signs, both pectins substantially suppressed allergen-specific IgE, the pivotal inducer of symptoms in type 1 allergies, but had only a slight effect on sIgG1 and no effect on the serum levels of sIgG2a (Figure 43, Figure 55). Thus, it should be considered that besides the “classical” anaphylactic reaction, involving IgE, mast cells and FcεRI, an alternative pathway was found in rodents, in which IgG together with e.g. macrophages, granulocytes and platelets are important (Strait et al. 2002). It was shown, that the IgG subclasses IgG1, IgG2a and IgG2b were able to induce systemic anaphylaxis combined with mild to severe hypothermia in mice (Miyajima et al. 1997; Beutier et al. 2017). Thus, the unaffected levels of IgG found in this study might indicate involvement of IgG in the allergy-related symptoms in the mice.

Pectin intervention did not only alter the levels of allergen-specific antibodies, but also levels of total Ig. Interestingly, only HMP intervention enhanced serum levels of total IgA, compared to the allergic control, whereas no effect with LMP intervention was seen. This was already observed after HMP supplementation to healthy mice and could be confirmed in the allergic setting (Figure 44, Figure 56). This might be due to the altered gut microbiota with shift into the direction of *Bacteroides* that was induced especially by HMP intervention, as it was reported that *Bacteroides* species might induce IgA production (Yang et al. 2020; Westrin et al. 1991). As IgA is associated with a lower risk for FAs due to inhibitory mechanisms, dietary intervention with HMP might provide beneficial effects in the context of FAs (Shamji et al. 2021; El Ansari et al. 2022).

In contrast, levels of total IgG were not affected by the pectin interventions in comparison to the allergic control, whereas they were enhanced in all PE-immunized groups, compared to the PBS control. This supports the hypothesis, that an IgG-mediated reaction might play a role in the development of allergy-related reactions in the mice that was not suppressed by pectin intervention. Levels of total IgE were slightly decreased by both pectin interventions, compared to the allergic control which fits to the reduced levels of sIgE observed in both pectin interventions. Intervention with both pectins showed similar effects on the total as well as sIgE levels, whereas primary-prophylactic interventions seemed to have a stronger impact. This observation might be due to the required re-direction of the established Th2-biased immune response with secondary-prophylactic intervention, whereas primary-prophylactic pectin intervention may suppress already the manifestation of the allergic reaction (O'Konek et al. 2018). Furthermore, both pectins strongly suppressed mast cell activation, measured as levels of mMCPT-1, whereas the first intervention experiment showed stronger effects by primary-prophylactic HMP intervention. This implicates that not only the pectin structure but also the time and duration of dietary intervention might play a role in suppressing allergic reactions.

Furthermore, pectin intervention modulated the composition of the gut microbiome, as a strong shift towards *Bacteroides* was observed, visible 4 days after start of the pectin diet. This shift could be observed with both structurally different pectins, whereas HMP induced a stronger modulation. Due to their high variety of degrading enzymes, *Bacteroides* are well-known as pectin-degraders which are able to respond quickly to the presence of polysaccharides in the environment (Ndeh and Gilbert 2018; Chung et al. 2017). *Bacteroides* are considered as beneficial bacteria for the host, as they are able to improve the host immunity, and maintain the gut microecological balance by production of SCFAs, especially acetate and propionate (Cheng et al. 2022; Shimizu et al. 2018). Interestingly, in the first intervention experiment the frequency of *Akkermansia* decreased in the groups after start of the pectin intervention. *Akkermansia* are described as mucus degrading bacteria that might therefore also be involved in manifestation of allergic reactions as the mucus barrier is an important factor to maintain tolerance (Rodrigues et al. 2022). However, this effect was not observed in the second intervention experiment, comparing both

pectins. It should be pointed out, that the gut microbiome of the mice is highly variable and may differ at initiation of the study, which might explain the observed differences in the results. Nevertheless, as this reflects the conditions and differences observed in humans and allergic patients it is of particular interest that both pectins and especially HMP were able to shift the microbiome towards *Bacteroides* in healthy as well as allergic conditions.

Interestingly, only HMP intervention decreased the microbial diversity, whereas no effect on Chao1 was observed with LMP, which is in line with previously reported effects upon *in vitro* fermentation (Larsen et al. 2019; Nakamura et al. 2023). Pectins are mainly degraded by members of the *Bacteroides* genera whereas their breakdown substrates produced during fermentation can be used by surrounding bacteria as growth substrate (Flint et al. 2007). Depending on the chemical structure, different breakdown products occur that can be used by certain bacterial strains. In this way the structure of pectin can determine the composition and richness of the microbial community. Due to the more complex structure of HMP, higher variety of enzymes is necessary for its fermentation, which limits the number of bacterial strains that can use HMP as growth substrate (Scott et al. 2014).

Furthermore, pectin intervention revealed several effects on the local immune response in the small and large intestine, as observed by the variations in the frequency of immune cells in the intestinal LP. Intervention with HMP as well as LMP reduced the frequencies of mast cells, macrophages and slightly of eosinophils in the small and the large intestine LP in comparison to the allergic control. In contrast, no or inconsistent effects were observed for neutrophils and cDCs. As neutrophils are strongly involved in inflammatory responses including allergic reactions, this suggests a beneficial role of both pectins by suppression and downregulation of the inflammatory immune response (Nakano and Kitaura 2022; Lombardi et al. 2022; Tordesillas et al. 2017b). This hypothesis is supported by the observed reduction of mMCPT-1 levels as well as levels of total IgE in the supernatant of small intestine homogenates.

Interestingly, different effects of pectin intervention were observed in the LP of the small or large intestine. This might be explained by the anatomical and also functional differences (e.g. mucus thickness, cellular heterogeneity or microbial

population) of small and large intestine that have been described as two separate immunological sites (Bowcutt et al. 2014). Whereas pectin degradation including production of mediators takes place mainly in the large intestine with a higher microbial density compared to the small intestine (Holloway et al. 1983; Pascale et al. 2022), sensitization to food allergens, local inflammatory responses as well as intestinal permeability mainly occur in the small intestine which is densely populated with immune cells (Ali et al. 2020). Thus, immune responses can be observed in both intestinal sites as due to immune cell migration induced by the gut microbiota, effects induced by dietary pectin and FA might impact both intestinal tracts (Ali et al. 2020).

In summary, this study showed that dietary pectin intervention was able to suppress allergy-related Th2 responses considering humoral and cellular responses. Both pectins led to decreased levels of sIgE, shifted the gut microbiota composition towards *Bacteroides* and modulated the local immune response analyzed by infiltrating cells in the LP. Furthermore, both pectins suppressed mast cell activation and reduced their abundance in the gut. Nevertheless, different chemical structures influenced the immune-modulatory capacity of the pectins, whereas the start of the dietary intervention seemed to play a minor role. Only apple-derived HMP revealed an impact on total IgA levels and affected the microbial richness. Furthermore, it is not known whether the effects observed with the two pectins are caused by modulations of the bacterial composition or induced at least partly by direct interaction with the immune cells. Further studies are required to fully understand the mechanisms underlying the immune-modulatory capacities of different pectins.

Finally, the obtained results generated evidence that dietary pectin intervention can beneficially modulate the immune response in healthy mice and – at least partially – suppress allergy-related immune responses in a model of FA, depending on the structural characteristics of the used pectin. This might implicate some truth in the common saying “an apple a day keeps the doctor away”. However, it might need more than an apple a day to obtain the modulatory effects observed in this study. Nevertheless, regular consumption of pectin-containing diet, including apples may maintain microbiota homeostasis, suppress inflammatory responses and beneficially affect FAs.

6 Summary

Food allergies (FAs) are defined as reproducible, adverse immunological reaction leading to allergic symptoms upon ingestion of certain food. With increasing prevalence worldwide, FAs are becoming a health as well as economic burden. Despite the increasing number of food allergic patients, there is no curative treatment available so far. Allergen-specific desensitizing approaches often do not lead to complete tolerance but instead might pose a risk for severe reactions and are only available for certain allergenic foods. Therefore, strict avoidance of the reactive food or symptomatic treatment including anti-histamines or epinephrine in severe cases are the options of choice for allergics.

FAs can be classified into IgE-mediated and non-IgE-mediated allergies, depending on the involved pathways and the role of IgE in the pathogenesis. IgE-mediated FAs are most common and can lead to symptoms of the gastrointestinal tract, the respiratory tract or the skin. In severe cases, however, anaphylactic reactions can occur, characterized by a generalized reaction, hypotension and anaphylactic shock. Mechanistically, FAs are a type I allergic reactions, consisting of a sensitization phase upon initial contact with the allergen and an effector phase after every following allergen ingestion. At the first contact with the allergen, it is taken up by antigen-presenting cells (APCs), such as dendritic cells (DC) and presented on their surface MHC class II receptors. This leads to activation and proliferation of naïve Th cells and following activation of B cells. Furthermore, activated B cells undergo class switch and produce allergen-specific IgE (sIgE) antibodies. These bind to the Fcε receptor I (FcεRI) on the surface of mast cells and basophils and lead to sensitization against the respective allergen. Upon a following contact to the allergen, cross-linking of the receptors is induced and it comes to release of different mediators that promote the inflammatory allergic reaction.

Among FAs, non-specific lipid transfer proteins (nsLTPs) play an important role as sensitizers to different fruits. These small, heat-stable and structurally highly conserved proteins can be found in a variety of plants, especially from the *Rosaceae* family. Due to the high homology of different nsLTPs patients often develop symptoms not only to one but to several nsLTPs, causing the “nsLTP syndrome”.

However, the peach nsLTP Pru p 3 is reported as one of the main sensitizers and reactivity can be detected for most of the nsLTP allergic patients.

A variety of extrinsic and intrinsic factors can promote the development and manifestation of FAs involving also individual genetic predispositions. However, in recent years dysbiosis of the microbiome gained focus as an important factor to promote allergies. Especially due to environmental exposure but also depending on nutritional habits, a dysregulation of the microbiome of the skin, lungs or gut can occur. Therefore, homeostasis of the microbiome and promotion of the activity and growth of certain beneficial bacteria, is suggested to prevent the development of allergies. Recent studies in this field focus on the effect of dietary fibers, especially prebiotics such as pectin or inulin, to modulate the microbiome in a beneficial way.

In general, dietary fibers are carbohydrates that cannot be digested by human enzymes in the small intestine, but can be fermented by different intestinal bacterial strains. Among the group of dietary fibers, prebiotics are categorized as non-digestible food ingredients that promote the activity and growth of certain bacteria and thereby improve the health of the host. It was reported that prebiotics, such as pectin can beneficially modulate the immune-response and thereby possibly suppress the development of allergic inflammation.

The aim of this study was to examine the effect of two structurally different pectins on the development and manifestation of peach allergy in a FA mouse model. Pectins are heteropolysaccharides that can be extracted mainly from peel and core of different fruits, including apple and citrus fruit. Due to the extraction method and the source material used, a high variability of the chemical structure can occur. For classification of pectins, the degree of esterification (DE) is used, distinguishing low methoxyl pectin (LMP) and high methoxyl pectin (HMP). It has been reported that pectins possess different immune-modulatory properties, either by potential direct interaction with intestinal and mucosal cells, or especially indirectly mediated by bacterial metabolites upon fermentation. Depending on their chemical structure, pectins are fermented more or less efficiently and thereby promote the growth of certain bacterial strains in the intestine. However, it is not known so far whether certain pectin structures exert distinct modulatory effects and if there is a certain pectin structure capable of promoting beneficial health effects. Therefore, the effect

of dietary supplementation of different pectins was examined in three healthy mouse strains. Thereby, modulation of the gut microbiome as well as immune-modulatory effects were examined in each of the mouse strains. To further study the impact of dietary pectin intervention on peach allergic mice, a mouse model of peach allergy was established. To achieve this, different sensitization and provocation routes were tested and the preparation of an allergen extract was optimized. In line with the study described here in a mouse model of peach allergy, a dietary intervention in peach allergic patients was conducted by collaboration partners.

In a first step, the two pectins used had to be characterized regarding potential traces of nsLTP that might pose a risk to the peach allergic patients in the dietary intervention study. As the pectins were extracted from apple and citrus fruit, which both are known to contain nsLTPs, the residual allergen content was examined. In this context, the nsLTP concentration that might induce oral allergic syndrome (OAS) or severe anaphylactic reaction was determined on the basis of threshold levels, defined by the clinical collaborators. To examine, whether these thresholds were detectable in the pectin samples, Pru p 3 – as a marker nsLTP – was spiked into the pectins at the concentrations relevant for eliciting allergic reactions. Immunoblot detection revealed a strong interference of the pectin matrices, making prior enzymatic treatment of the pectin samples necessary. Further precipitation of pectin or protein enabled detection of the allergen spiked in both pectins with no detectable allergen in the unspiked pectin sample. This could be confirmed using different detection methods as well as a mediator release assay with humanized rat basophil leukemia cells (huRBL). In summary, it was concluded that the potential residual amount of nsLTP in the pectins is below the thresholds and poses no risk to the peach allergics for development of severe reactions.

Next, the effect of dietary pectin supplementation was evaluated in naïve mice, comparing three different mouse strains (CBA/J, C57BL/6 and BALB/c). Therefore, 5% or 15% of HMP or LMP (adjusted to 20% with cellulose) were supplemented to the mouse diet for 14 days and compared to a control diet containing 20% cellulose. Analysis of the fecal microbiota composition during the feeding period revealed a reduction of the microbial richness after HMP supplementation. Also, a strong growth of *Bacteroides* was induced especially by HMP (5% and 15%)

supplementation in all three mouse strains, which was also observed with 15% LMP. HMP also exerted a strong immune-modulatory effect, increasing levels of total IgA and IgG in the serum and proportionally lowering the levels of total IgE. In comparison, LMP supplementation only slightly increased levels of total IgA in CBA/J mice. Additionally, pectin supplementation affected the intestinal physiology, measured as length of intestinal sections, as well as frequencies of infiltrating cells in the lamina propria (LP). The most prominent effects were observed with HMP containing diet, leading to strong enlargement of the caecum and decrease of several immune cells in the LP, including mast cells. Pectin supplementation to naïve mice revealed stronger effects when HMP was supplemented compared to LMP. This leads to the conclusion that the modulatory capacity, affecting microbial composition as well as immune responses strongly depends on the pectin structure.

To examine the immune-modulatory effects of pectin in a model of FA, a peach allergy mouse model was established. Due to prior experiments performed in our group, CBA/J mice were used due to development of a strong Th2-biased immune reaction upon nsLTP sensitization. Different sensitization and provocation routes, using either peach peel extract (PE) or natural Pru p 3 (nPru p 3) were tested and compared regarding development of allergy-related symptoms and immune reaction. In the final model, mice were sensitized by intraperitoneal (i.p.) injection, using PE and aluminum hydroxide (alum) as adjuvant, followed by oral administration of PE and a final i.p. provocation with PE. This schedule led to a massive drop in body temperature as well as high levels of allergen-specific IgE (sIgE), sIgG1 and sIgG2a. Furthermore, levels of murine mast cell protease-1 (mMCPT-1) were increased, indicating activation of mast cells and development of a Th2-driven immune reaction in the mice. In addition, local immune responses were observed in the LP of the small intestine, such as increased frequencies of immune cells or antibody levels, indicating the involvement of the gastrointestinal tract in the model.

Finally, the effect of dietary pectin intervention was determined in peach allergic mice using the established peach allergy mouse model. Two independent experiments were performed: I) dietary intervention with 15% HMP in a primary-prophylactic (start of pectin diet before sensitization) and secondary-prophylactic

(start of pectin diet after sensitization) approach and II) dietary intervention with 15% HMP or 15% LMP in primary-prophylactic and secondary-prophylactic approach. Both experiments revealed only a slight effect of pectin on the development of allergy-related clinical signs. However, both pectins were able to decrease the levels of sIgE in the serum. In line with the results obtained in healthy mice, both pectins, modulated the gut microbiota into the direction of *Bacteroides* but only HMP decreased the microbial richness. Both pectins, but especially HMP intervention affected the frequency of the infiltrating cells in the LP, leading e.g. to a strong decrease of mast cells in the intestine.

Taken together, this study could demonstrate that I) the two used pectins provide no risk for nsLTP allergic patients, as the amount of potential residual nsLTP is below the thresholds to induce severe allergic reactions. II) structurally different pectins exert distinct modulatory effects on the microbiome and the immune response. Both pectins modulated the microbiota composition in the gut, whereas only HMP affected the microbial richness. Furthermore, mainly HMP supplementation altered the immune response in naïve mice. III) HMP and LMP were able to decrease the levels of sIgE in peach allergic mice and to modulate the frequencies of infiltrating cells in the LP. Stronger effects could be observed when HMP compared to LMP was used for dietary intervention.

This study helped to gain insights into the immune-modulatory effects of different pectins on FA by modulation of the gut microbiota. It supports the hypothesis, that the structural properties play an important role for the modulatory capacities of the different pectins. HMP (5% and 15%) showed stronger modulatory effects on the humoral immune response as well as on the gut microbiota composition compared to LMP, whereas only 15% LMP was capable to induce immune modulation. However, it is not known whether the effects observed with the two pectins are due to differences in the fermentability and metabolite production by bacterial strains or direct interaction with the immune cells. Furthermore, not only the DE might play a role but also other structural factors, such as amount of GalA or type and number of branches. Therefore, it might still be interesting, to further elaborate the modulatory effects of distinct chemical structures on immune response and microbiota composition.

7 Zusammenfassung

Nahrungsmittelallergien sind reproduzierbare Immunreaktionen, die nach der Aufnahme bestimmter Lebensmittel auftreten und allergische Symptome auslösen. Da die Prävalenz für Nahrungsmittelallergien weltweit ansteigt, stellen diese eine große gesundheitliche sowie ökonomische Belastung dar. Trotz der zunehmenden Zahl an betroffenen Patienten gibt es zum aktuellen Zeitpunkt keine Therapie- oder Heilungsmöglichkeit. Allergenspezifische Desensibilisierungen führen häufig nicht zu kompletter Toleranz, sondern stellen ein Risiko für schwere allergische Reaktionen dar. Außerdem steht diese Therapieform nur für wenige Allergene zur Verfügung. Daher ist die strikte Vermeidung der allergieauslösenden Lebensmittel sowie symptomatische Behandlung nach versehentlicher Exposition mittels Antihistaminikums oder Epinephrin in schweren Fällen das Mittel der Wahl.

Immunologisch können Nahrungsmittelallergien in IgE-induzierte und nicht-IgE-induzierte Allergien unterteilt werden, je nachdem welche immunologischen Reaktionen involviert sind. IgE-induzierte Nahrungsmittelallergien stellen dabei die häufigste Form dar und können zu Symptomen im Magen-Darm-Trakt, im respiratorischen System sowie der Haut führen. In schweren Fällen kann es zu anaphylaktischen Reaktionen kommen, die durch eine generalisierte Reaktion, Hypotension und Schock gekennzeichnet sind. Mechanistisch gehören Nahrungsmittelallergien zu Typ I allergischen Reaktionen, bestehend aus einer Sensibilisierungs-Phase beim initialen Kontakt mit dem Allergen und einer Effektor-Phase nach jedem weiteren Kontakt. Bei erstmaligem Kontakt mit dem Allergen wird dieses von antigenpräsentierenden Zellen, wie beispielsweise dendritischen Zellen aufgenommen und auf deren MHC Klasse II Rezeptoren auf der Oberfläche präsentiert. Dies führt zur Aktivierung und Proliferation naïver Th Zellen und nachfolgender Aktivierung von B Zellen. Im Weiteren unterlaufen die aktivierten B Zellen einen Klassenwechsel zur Produktion von Allergen-spezifischen IgE (sIgE) Antikörpern. Diese binden an den Fcε-Rezeptor I (FcεRI) auf der Oberfläche von Mastzellen und Basophilen und führen somit zur Sensibilisierung gegen dieses Allergen. Bei wiederholtem Kontakt mit dem Allergen kommt es zur Kreuzvernetzung der Rezeptoren und folglich zur Ausschüttung diverser Mediatoren und Entstehung der Entzündungsreaktion.

7 Zusammenfassung

Unter den Nahrungsmittelallergien spielen nicht-spezifische Lipidtransferproteine (nsLTPs) eine wichtige Rolle bei der Sensibilisierung gegen verschiedene Früchte. Die kleinen, hitzestabilen und hochkonservierten Proteine kommen in einer Vielzahl von Pflanzen, besonders bei Vertretern der *Rosaceae* Familie vor. nsLTPs besitzen eine stark homologe Struktur, wodurch Allergiker häufig Symptome gegen eine Vielzahl von nsLTP entwickeln, was zum sogenannten „nsLTP Syndrom“ führt. Das Pfirsich-Allergen Pru p 3 stellt ein Hauptallergen bei der Sensibilisierung gegen nsLTPs dar, da viele nsLTP-Allergiker Sensibilisierung gegen Pru p 3 aufweisen.

Eine Vielzahl an Faktoren kann bei der Entstehung und Manifestation von Nahrungsmittelallergien eine Rolle spielen. Dazu gehören bspw. genetische Prädisposition sowie epigenetische Einflüsse. In den letzten Jahren ist auch Dysbiose des Mikrobioms als möglicher begünstigender Faktor für Allergien in den Fokus gerückt. Vor allem Umwelteinflüsse, aber auch Essgewohnheiten können zu einer Dysbiose des Mikrobioms in Darm, Lunge oder Haut führen. Studien zeigten hingegen, dass Homöostase des Mikrobioms die Entstehung von Allergien verhindern kann, weshalb aktuellen Studien in diesem Gebiet vor allem den Einfluss von Ballaststoffen und Präbiotika auf Allergien untersuchen, da diese das Mikrobiom positiv beeinflussen können.

Ballaststoffe sind Kohlenhydrate die vom Menschen nicht verdaut werden können, jedoch von verschiedenen Darmbakterien verstoffwechselt werden. Innerhalb der Gruppe der Ballaststoffe, werden Präbiotika als unverdauliche Lebensmittelbestandteile definiert, die die Aktivität und das Wachstum bestimmter Bakterien fördern und dabei gesundheitsfördernde Effekte haben können. Es konnte gezeigt werden, dass Präbiotika wie Pektine, eine modulatorische Wirkung auf die Immunantwort haben und eventuell die Entstehung allergischer Entzündungsreaktionen unterbinden können.

Das Ziel dieser Studie war es, die immunmodulierenden Eigenschaften zweier strukturell unterschiedlicher Pektine hinsichtlich Entstehung und Manifestation von Pfirsichallergie in einem Mausmodell zu untersuchen. Pektine sind Heteropolysaccharide die vor allem aus der Schale und dem Kerngehäuse verschiedener Früchte, bspw. Apfel oder Zitrusfrucht gewonnen werden können. Je nach Extraktionsmethode und Ausgangsmaterial kann die chemische Struktur der

Pektine variieren. Zur Klassifizierung, wird der Veresterungsgrad (DE) der Pektine verwendet und man unterscheidet zwischen niederveresterten (LMP) und hochveresterten Pektinen (HMP). Es konnte gezeigt werden, dass die durch Pektin induzierten immunmodulierenden Effekte entweder durch direkte Interaktion mit Epithelzellen hervorgerufen werden, oder indirekt durch Fermentierung durch Darmbakterien und Bildung verschiedener Metabolite. Pektine werden je nach chemischer Struktur unterschiedlich fermentiert und fördern daher das Wachstum unterschiedlicher Darmbakterien, wobei aktuell nicht bekannt ist, welche Pektin-Struktur die optimalen gesundheitsfördernden Effekte bewirkt. Daher wurde in dieser Studie die Supplementierung strukturell unterschiedlicher Pektine an gesunde Mäuse unterschiedlicher Mausstämme und der Effekt auf Immunantwort und Mikrobiom untersucht. Um im Weiteren den Effekt von Pektinen auf Pfirsichallergie zu untersuchen, wurde ein Pfirsichallergie Mausmodell etabliert. Dafür wurden verschiedene Applikationswege für die Sensibilisierung und Provokation getestet sowie die Herstellung eines Pfirsichextrakts (PE) standardisiert. Parallel zur hier dargestellten Studie in pfirsichallergischen Mäusen wurde von Kollaborationspartnern eine diätetische Pektin-Intervention in Pfirsichallergikern durchgeführt.

Zuerst wurden die beiden verwendeten Pektine bezüglich möglicher Restallergenbestände charakterisiert. Durch die Gewinnung der Pektine aus Äpfeln oder Zitrusfrüchten, die ebenfalls nsLTPs enthalten, könnten Allergen-Rückstände ein Risiko für Pfirsichallergiker darstellen. Es wurden Schwellenwerte für die Entstehung des oralen Allergiesyndroms (OAS) oder anaphylaktischer Reaktionen definiert. Um herauszufinden, ob die berechneten Schwellenwerte in den Pektinen nachweisbar sind, wurde Pru p 3 als Marker-nsLTP den Pektinproben zugesetzt. Nachweis mittels Immunoblot zeigte eine starke Interferenz durch die Pektinmatrix, weshalb es nötig war die Pektinproben zuvor enzymatisch zu verdauen. Außerdem konnte mittels Fällung von Pektin bzw. Protein die Sensitivität erhöht werden und das zugegebene Allergen war in beiden Pektinproben nachweisbar. In Pektin ohne zugegebenem Pru p 3 konnte kein nsLTP nachgewiesen werden. Dies konnte durch einen weiteren funktionellen zellbasierten Test bestätigt werden, wodurch

geschlussfolgert werden konnte, dass möglicherweise enthaltene nsLTP-Rückstände kein Risiko für nsLTP-Allergiker darstellen.

Im nächsten Schritt wurde der Einfluss von Pektin auf gesunde Mäuse dreier unterschiedlicher Mausstämme (CBA/J, C57BL/6 und BALB/c) untersucht. Es wurden entweder 5% oder 15% LMP oder HMP für 14 Tage in den Futterpellets verabreicht. Anschließende Untersuchung der Fäzesproben zeigte, dass vor allem HMP das Wachstum von Bakterien der Gattung *Bacteroides* in allen drei Mausstämmen förderte. Außerdem zeigte vor allem Zugabe von HMP immunmodulierende Effekte, wie u.a. erhöhte Serumlevel von IgA und IgG, sowie einen Anstieg des IgG/IgE Verhältnisses. Des Weiteren beeinflusste Pektin die Darmphysiologie, die durch Messung verschiedener Darmabschnitte sowie Analyse der infiltrierenden Zellen der Lamina propria (LP) charakterisiert wurde. HMP-angereichertes Futter erzielte hierbei die stärksten Effekte und führte bspw. zu einer Reduktion von Mastzellen. Diese Ergebnisse zeigten, dass der Einfluss auf das Mikrobiom sowie die Immunantwort von den strukturellen Eigenschaften der Pektine abzuhängen scheint.

Um den Einfluss von Pektinen nicht nur in gesunden Mäusen, sondern auch im Kontext der Pfirsichallergie zu untersuchen, wurde ein Pfirsichallergie Mausmodell etabliert. Basierend auf vorherigen Experimenten, wurden dafür CBA/J Mäuse gewählt, da diese eine starke Th2-gerichtete Reaktion auf nsLTP-Sensibilisierung entwickeln. Verschiedene Applikationswege für Pru p 3 oder Pfirsichextrakt wurden getestet und hinsichtlich der Entstehung von Symptomen und Immunantwort verglichen. Für das etablierte Modell wurden die Mäuse intraperitoneal (i.p.) mit PE sensibilisiert, anschließend wurde PE oral verabreicht und die Provokation wurde i.p. mit PE durchgeführt. Dies führte zu einem starken Abfall der Körpertemperatur sowie hohen allergen-spezifischen IgE (sIgE), sIgG1 und sIgG2 Leveln. Auch Level der murinen Mastzellprotease-1 (mMCPT-1) waren stark erhöht, was auf eine Aktivierung der Mastzellen und Th2-gerichtete Immunantwort hindeutet. Es konnten außerdem lokale Reaktionen, wie erhöhte Zahl der Immunzellen in der LP, beobachtet werden.

Anschließend wurde der Effekt der Pektine auf pfirsichallergische Mäuse untersucht. Dafür wurden zwei unabhängige Experimente durchgeführt:

I) Diätetische Intervention mit 15% HMP als primär-prophylaktischer (Beginn der Pektin-Intervention vor Sensibilisierung) oder sekundär-prophylaktischer Ansatz (Beginn der Pektin-Intervention nach Sensibilisierung), sowie II) diätetische Intervention mit 15% HMP oder LMP als primär- und sekundär-prophylaktischer Ansatz. In beiden Experimenten konnte Pektin die Entstehung von Symptomen nach Provokation nicht verhindern. Beide Pektine führten jedoch zu einer starken Reduktion der sIgE Level. Außerdem modulierten beide Pektine das Darmmikrobiom in Richtung *Bacteroides*, wobei nur HMP zu einer Reduktion der mikrobiellen Diversität führte. Die infiltrierenden Immunzellen in der LP wurden durch beide Pektine, jedoch vor allem durch HMP, beeinflusst und bspw. die Anzahl der Mastzellen in der LP reduziert.

Zusammenfassend konnte diese Studie zeigen, dass I) die beiden verwendeten Pektine kein Risiko für nsLTP-Allergiker darstellen, da mögliche Allergen-Restbestände unter den Schwellenwerten für die Auslösung allergischer Reaktionen liegen. II) Strukturell unterschiedliche Pektine das Mikrobiom und die Immunantwort in unterschiedlicher Weise modulieren, wobei der stärkste Einfluss durch HMP beobachtet wurde und nur HMP einen Einfluss auf die mikrobielle Diversität hatte. Dies konnte in gesunden sowie allergischen Mäusen beobachtet werden. III) HMP und LMP die sIgE Level in pfirsichallergischen Mäusen senkten und die Ig-Level im Serum beeinflussten. Außerdem modulierten die Pektine die Anzahl der infiltrierenden Zellen in der LP. Dies konnte mit beiden Pektinen beobachtet werden, war jedoch stärker, wenn HMP zur diätetischen Intervention verwendet wurde.

Diese Studie konnte dazu beitragen, weitere Erkenntnisse zu den immunomodulatorischen Eigenschaften von Pektinen in gesunden sowie allergischen Mäusen zu gewinnen. Sie stützt die Hypothese, dass die Struktur verschiedener Pektine eine wichtige Rolle für deren modulierende Eigenschaften spielt. Hochverestertes (HMP) Pektin zeigte einen größeren Einfluss auf die Zusammensetzung des Darmmikrobioms sowie diverse Immunreaktionen, im Vergleich zu niederverestertem (LMP) Pektin. Ob diese Effekte jedoch durch direkte Interaktion mit den Epithel- und Immunzellen oder indirekt mittels Fermentation durch Darmbakterien und Bildung diverser Mediatoren ausgelöst werden, ist weiterhin unklar und Fragestellung für weiterführende Studien.

8 Supplementary

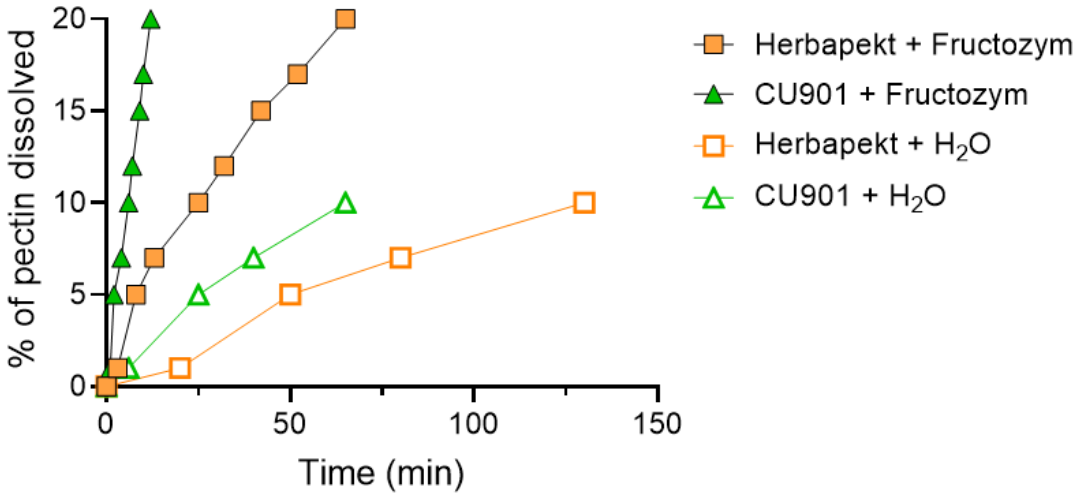

Figure S1: Solubility of different pectins in Fructozym-solution or water.
Solubility of the two different pectins was determined over time in water or a pectinase (Fructozym)-solution up to 150 min.

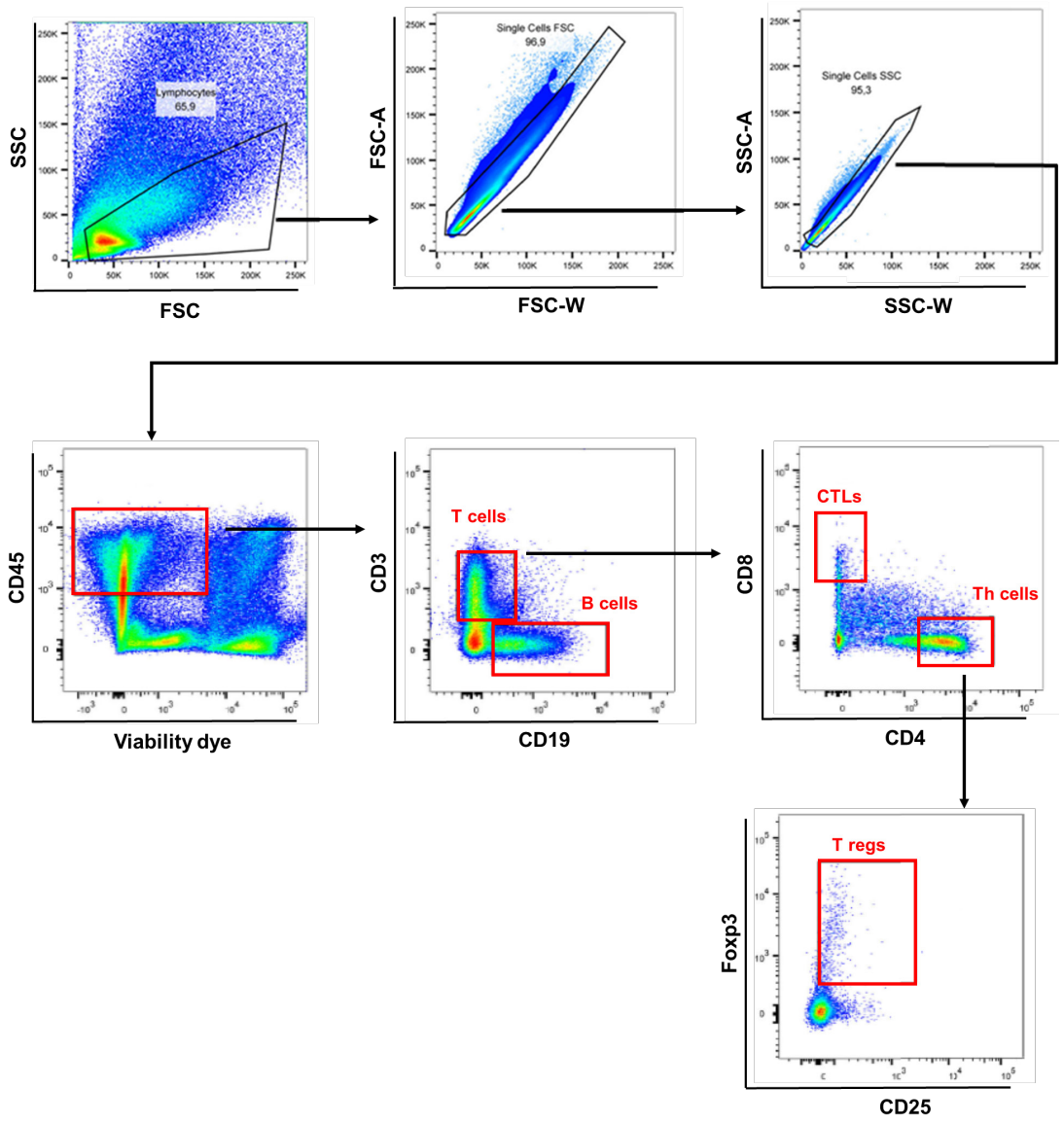

Figure S2: Gating strategy T and B cell panel.
Live lymphocytes were gated for FSC, SSC characteristics, cleaned up from doublets in FSC and SSC channel and identified as $CD45^+$ live population. Among alive $CD45^+$ cells, B cells were identified as $CD19^+CD3^-$ and T cells as $CD19^-CD3^+$ populations. T cells were further discriminated for $CD8^+$ cytotoxic T cells (CTLs) or $CD4^+$ T helper cells (Th). Regulatory T cells (Tregs) were identified as $Foxp3^+$ population among $CD4^+$ cells.

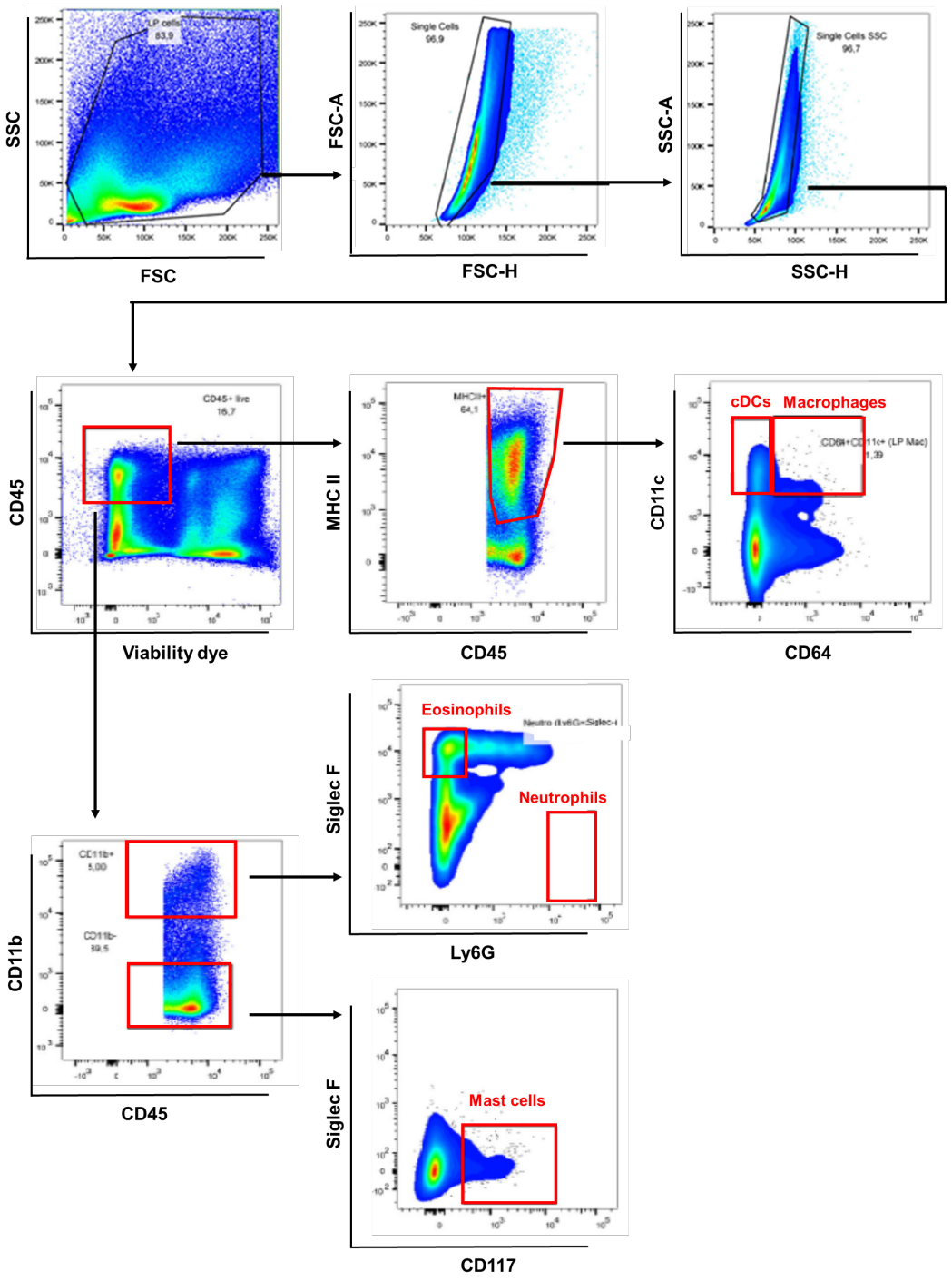

Figure S3: Gating strategy granulocyte panel.
Live lamina propria cells were gated for FSC, SSC characteristics, cleaned up from doublets in FSC and SSC channel and identified as CD45$^+$ live population. Among these, CD11b$^+$ populations were identified. Among CD11b$^+$ population, eosinophils were SiglecF$^+$Ly6G$^+$ and neutrophils SiglecF- and Ly6G$^+$. Mast cells were identified as CD117$^+$SiglecF- among CD11b$^-$ population. In addition, MHCII$^+$ cells were identified among CD45$^+$ cells and subsequently analyzed for CD11c$^+$CD64$^-$ conventional dendritic cells (cDCs) and CD11c$^-$ CD64$^+$ macrophages.

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Declaration of academic integrity

Except where stated otherwise by reference or acknowledgment, the work presented was generated by myself under the supervision of my advisors during my doctoral studies. All contributions from colleagues are explicitly referenced in the thesis.

The material listed below was obtained in the context of collaborative research:

Figure 17: Effect of pectin on microbial richness and composition in CBA/J mice. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany) performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 18: Beta diversity after supplementation of pectin to CBA/J mice. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany) performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 19: Effect of pectin on microbial richness and composition in C57BL/6 mice. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany) performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 20: Beta diversity after pectin supplementation in C57BL/6 mice. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany) performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 21: Pectin supplementation affects microbial richness and composition in BALB/c mice. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany) performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 22: Beta diversity in BALB/c mice after pectin supplementation. Dr. Dominik Stoll and Dr. Melanie Huch (Max Rubner-Institut, Karlsruhe, Germany)

performed DNA isolation, sequencing, bioinformatic and statistical analysis. My contribution: execution of mouse experiments and collection of sample material.

Figure 26: Histological analysis of jejunum after pectin supplementation. Dr. Gonzalez-Menendez and Dr. Leticia Quintanilla-Martinez (Eberhard Karls University, Tübingen, Germany) performed H&E staining and data analysis and interpretation. My contribution: execution of mouse experiments including collection and provision of sample material.

Figure 46: Effect of HMP intervention on microbial .
Dr. Csaba Miskey (Paul-Ehrlich-Institut, Langen, Germany) performed DNA sequencing and Dr. Oleg Krut (Paul-Ehrlich-Institut, Langen, Germany) did bioinformatic and statistical analysis of the data. My contribution: execution of mouse experiments, collection of sample material, DNA isolation and quantification, Amplicon- and Index-PCR

Figure 47: Modulation of gut microbiota composition by HMP intervention. Dr. Csaba Miskey (Paul-Ehrlich-Institut, Langen, Germany) performed DNA sequencing and Dr. Oleg Krut (Paul-Ehrlich-Institut, Langen, Germany) did bioinformatic and statistical analysis of the data. My contribution: execution of mouse experiments, collection of sample material, DNA isolation and quantification, Amplicon- and Index-PCR

Figure 59: Effect of HMP or LMP intervention on microbial richness. Dr. Csaba Miskey (Paul-Ehrlich-Institut, Langen, Germany) performed DNA sequencing and Dr. Oleg Krut (Paul-Ehrlich-Institut, Langen, Germany) did bioinformatic and statistical analysis of the data. My contribution: execution of mouse experiments, collection of sample material, DNA isolation and quantification, Amplicon- and Index-PCR

Figure 60: Modulation of gut microbiota composition by HMP or LMP intervention. Dr. Csaba Miskey (Paul-Ehrlich-Institut, Langen, Germany) performed DNA sequencing and Dr. Oleg Krut (Paul-Ehrlich-Institut, Langen, Germany) did bioinformatic and statistical analysis of the data. My contribution: execution of mouse experiments, collection of sample material, DNA isolation and quantification, Amplicon- and Index-PCR

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The following parts of the thesis haven been previously published:

- Chapter 4.1 “Risk assessment of pectin”
- Chapter 4.3.1 “Characterization of peach peel extract”
- Chapter 4.3.5 “Characterization of the established peach allergy mouse model”
- Figure 9 “nsLTP detection in HMP or LMP by immunoblot”
- Figure 10 “Detectability of nsLTPs in pectin matrix by ELISA”
- Figure 11 “IgG-reactivity of nPru p 3 after enzymatic treatment”
- Figure 12 “nsLTP detection in pectin upon enzymatic treatment”
- Figure 13 “Detection of nsLTP thresholds in HMP and LMP”
- Figure 14 “Protein or pectin precipitation increases nsLTP detectability”
- Figure 15 “Detection of nsLTP thresholds by mediator release assay”
- Figure 32 “Confirmation of temperature drop and development of clinical signs”
- Figure 33 “Induction of humoral immune response by PE”
- Figure 34 “mMCPT-1 levels and IgG reactivity of mouse serum”
- Figure 35 “Total immunoglobulin levels measured in the serum of peach allergic mice”
- Figure 36 “Measurement of cytokine secretion in peach allergic mice”
- Figure 37 “Measurement of intestine length in peach allergic mice”
- Figure 38 “Infiltrating immune cells in the small intestine lamina propria of peach allergic mice”
- Figure 39 “mMCPT-1 and total Ig levels in the intestine of peach allergic mice”

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