Carbohydrate Polymers 334 (2024) 122007

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effects of pectin methyl-esterification on intestinal microbiota and its immunomodulatory properties in naive mice

Hanna Steigerwald [a,1], Frank Blanco-Pérez [a,1], Andrea Macías-Camero [b,c], Melanie Albrecht [a], Melanie Huch [d], Caroline Bender [e], Stefan Schülke [a], Judith Keller [e], Maren Krause [a], Coral Barbas [c], Irene Gonzalez-Menendez [f,g], Leticia Quintanilla-Martinez [f,g], Masako Toda [h], Domingo Barber [b], Sabine Kulling [d], Mirko Bunzel [e], Stefan Vieths [a], Alma Villaseñor [b,c], Dominic Stoll [d], Stephan Scheurer [a,*]

[a] Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Molecular Allergology, 63225 Langen, Germany
[b] Departmento de Ciencias Médicas Básicas, Instituto de Medicina Molecular Aplicada (IMMA) Nemesio Díez, Facultad de Medicina, Universidad San Pablo-CEU, CEU Universities, 28660 Boadilla del Monte, Spain
[c] Centro de Metabolómica y Bioanálisis (CEMBIO), Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, 28660 Boadilla del Monte, Spain
[d] Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Fruit and Vegetables, 76131 Karlsruhe, Germany
[e] Karlsruhe Institute of Technology, Institute of Applied Biosciences, Department of Food Chemistry and Phytochemistry, 76131 Karlsruhe, Germany
[f] Eberhard Karls University of Tübingen, Institute of Pathology and Neuropathology, Comprehensive Cancer Center, 72074 Tübingen, Germany
[g] Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", Eberhard Karls University of Tübingen, 72074 Tübingen, Germany
[h] Laboratory of Food and Biomolecular Science, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-8577, Japan

ARTICLE INFO

Keywords:
Pectin esterification
High methoxyl pectin
Low methoxyl pectin
Polygalacturonic acid
Naïve mice
Intestinal microbiota

ABSTRACT

Pectins are dietary fibers that are attributed with several beneficial immunomodulatory effects. Depending on the degree of esterification (DE), pectins can be classified as high methoxyl pectin (HMP) or low methoxyl pectin (LMP).

The aim of this study was to investigate the effects of pectin methyl-esterification on intestinal microbiota and its immunomodulatory properties in naive mice.

Supplementation of the diet with LMP or HMP induced changes in the composition of the intestinal microbiota in mice toward *Bacteroides*, which was mainly promoted by HMP. Metabolome analysis of stool samples from pectin-fed mice showed a different effect of the two types of pectin on the levels of short-chain fatty acids and

Abbreviations: Ara, arabinose; ASV, amplicon sequence variant; BA(s), bile acid(s); DE, degree of esterification; ESI, electrospray ionization; Gal, galactose; Glc, glucose; HMP, high methoxyl pectin; HPAEC, high performance anion exchange chromatography; JSD, Jensen-Shannon divergence; LMP, low methoxyl pectin; Mal, maltose; Man, mannose; MS (MS^2), mass spectrometry (tandem MS); MVA, multivariate analysis; PAD, pulsed amperometric detection; PLS-DA, partial least squares-discriminant analysis; QqQ-MS, triple quadrupole MS; Rha, rhamnose; SCFA(s), short-chain fatty acid(s); SEM, standard error of the mean; SPF, specific pathogen free; UHPLC, ultra-high-performance liquid chromatography; VIP, Variable Importance in the Projection; Xyl, xylose; ACN, acetonitrile; CA, cholic acid; CA-d4, Cholic Acid-d4; CDCA, Chenodeoxycholic acid; CDCA-d4, Chenodeoxycholic Acid-d4; 5-cholenic, 5-cholenic acid-3β-ol; DCA, deoxycholic acid; 3ß-DCA, 5β-cholanic acid-3β, 12α-diol; DCA-d4, Deoxycholic Acid-d4; 7-KLCA, 3α-hydroxy-7 ketolithocholic acid; 3,7,12-DHCA, 3,7,12 dehydrocholic acid; DnsHz, dansylhydrazine; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; GalA, galacturonic acid; GCA-d4, Glycocholic Acid-d4; 3,7,12-GDHCA, 3,7,12 Glycodehydrocholic acid; HOAT, 1-hydroxy-7-azabenzotriazole; iLCA, isolithocholic acid; LCA, lithocholic acid; β-MCA, β-muricholic acid; MDCA, murocholic acid; IPA, 2-propanol; TαMCA, tauro-α muricholic acid; TUDCA, tauro-ursodeoxycholic acid; TCA, taurocholic acid; UDCA, ursodeoxycholic acid; 3,7,12-TDHCA, 3,7,12 taurodehydrocholic acid; UDCA-d4, Ursodeoxycholic Acid-d4.

* Corresponding author at: Molecular Allergology, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Molecular Allergology, Langen, Germany.
E-mail addresses: hanna.st92@gmx.de (H. Steigerwald), febp84@gmail.com (F. Blanco-Pérez), andrea.maciascamero1@ceu.es (A. Macías-Camero), Melanie.albrecht@pei.de (M. Albrecht), melanie.huch@mri.bund.de (M. Huch), caroline.bender@kit.edu (C. Bender), Stefan.Schuelke@pei.de (S. Schülke), Judith.keller@kit.edu (J. Keller), maren.krause@pei.de (M. Krause), cbarbas@ceu.es (C. Barbas), Irene.Gonzalez-Menendez@med.uni-tuebingen.de (I. Gonzalez-Menendez), Leticia.Quintanilla-Fend@med.uni-tuebingen.de (L. Quintanilla-Martinez), masako.toda.a7@tohoku.ac.jp (M. Toda), domingo.barberhernandez@ceu.es (D. Barber), sabine.kulling@mri.bund.de (S. Kulling), mirko.bunzel@kit.edu (M. Bunzel), Stefan.vieths@pei.de (S. Vieths), alma.villasenor@ceu.es (A. Villaseñor), dominic.stoll@mri.bund.de (D. Stoll), Stephan.scheurer@pei.de (S. Scheurer).
[1] Equally contributed.

https://doi.org/10.1016/j.carbpol.2024.122007
Received 13 December 2023; Received in revised form 22 February 2024; Accepted 27 February 2024
Available online 5 March 2024

bile acids, which was consistent with highly efficient *in vivo* fermentation of LMP. Analysis of serum antibody levels showed a significant increase in IgG and IgA levels by both pectins, while FACS analysis revealed a decrease of infiltrating inflammatory cells in the intestinal lamina propria by HMP.

Our study revealed that the structural properties of the investigated pectins determine fermentability, effects on microbial composition, metabolite production, and modulation of immune responses. Consumption of HMP preferentially altered the gut microbiota and suppressed pro-inflammatory immune responses, suggesting a beneficial role in inflammatory diseases.

1. Introduction

The dietary fiber pectin consists of a complex hetero-polysaccharide structure and is an important component of cell walls and intercellular compartments of higher plants. Due to their physicochemical characteristics, pectins are widely used as gelatinizer, thickener, stabilizer, or fat replacer in food industry for the production of *e.g.* jams, yoghurt, or ice cream (Tan & Nie, 2020). For commercial production, pectins are mainly extracted from peel and pulp of fruits or vegetables - including apple, citrus fruits, and sugar beet - by acidic extraction followed by alcoholic precipitation (Canteri-Schemin, Fertonani, Waszczynskyj, & Wosiacki, 2005; Larsen et al., 2019).

The chemical structure of the pectins can vary depending on the source material and the preparation method. In general, the linear unbranched backbone of pectins ("smooth region") is composed of homogalacturonan (HG), which consists of α-(1,4)-linked-D-galacturonic acid (GalA) units. These units can be acetylated at the C_2/C_3 hydroxyl groups or methyl-esterified at the C_6 carboxyl group (Dranca & Oroian, 2018). Furthermore, branched ("hairy") regions exist, that are dominated by rhamnogalacturonan-I (RG-I) polymers. The RG-I backbone carries varying degrees of neutral side chains, such as galactans, arabinans or arabinogalactans (Sila et al., 2009). The typical molecular mass of commercial pectins is around 50–150 kDa (Dongowski, 1997). To classify different types of pectins, the degree of methyl-esterification (DE) is used, which describes the ratio of methyl-esterified GalA units to unmethylated GalA units. Accordingly, 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, Alkarkhi, & Easa, 2009).

Several dietary fibers, including pectin, are considered to provide health benefits such as improving physical bowel function (Schwartz, Levine, Singh, Scheidecker, & Track, 1982), maintaining normal blood cholesterol levels, reducing glycemic response after food intake (Flourie, Vidon, Florent, & Bernier, 1984), and controlling inflammatory processes (Sun et al., 2017). These pectin-induced health benefits can be either achieved by direct effects of the dietary fiber on immune cells or by indirect effects mediated by the gut microbiota upon fermentation (Blanco-Pérez et al., 2021; Larsen et al., 2019). It has been suggested that their immune modulating properties depend on structural characteristics of the dietary fiber polysaccharides (Firrman et al., 2022).

Pectins are dietary fibers that cannot be digested by human enzymes and which are discussed to have prebiotic properties (Pascale, Gu, Larsen, Jespersen, & Respondek, 2022) as they are mostly degraded in the intestine by commensal bacteria that promote the growth and colonization of some beneficial bacterial strains such as *Bifidobacteria*, *Lactobacilli*, or *Bacteroides* species (Gómez et al., 2014; Tingirikari, 2018). The degradation of pectin polysaccharides by bacterial enzymes at the outer membrane initially releases oligomeric intermediates. Further cellular metabolism of these cleavage products in the microbiota leads to the formation of short-chain fatty acids (SCFAs), metabolites which mediate immunomodulatory effects (El Kaoutari, Armougom, Gordon, Raoult, & Henrissat, 2013; Fukunaga et al., 2003) and trigger anti-inflammatory effects (Blaut, 2002; Gómez et al., 2014). By this, pectin consumption positively affects homeostasis of the gut microbiome (Hasan & Yang, 2019). In line with this, bile acids (BAs) are considered to be important metabolites of the cholesterol metabolism, acting as mediators between the host and its gut microbiota (Kiriyama & Nochi, 2021; Tian et al., 2020). Depending on the type of bile acid, they may have pro- or anti-inflammatory effects on the immune system, making their role particularly important in microbiota-related diseases (Kiriyama & Nochi, 2021). However, the effect of pectin on the production of BAs is not yet known.

It has been suggested that structural features of pectin such as DE in combination with the degree of blockiness of methyl-esterified GalA (Beukema et al., 2022; Tang et al., 2023), free carboxylic groups, molecular mass, distribution, and composition of side chains determine the effect of pectin on the immune system (Blanco-Pérez et al., 2021). Accordingly, HG backbones of pectin macromolecules might have immune-suppressive activity (Popov & Ovodov, 2013). Nevertheless, knowledge about the effect of different pectin structures on cellular and humoral immune responses is limited. Thus, it is not known whether pectin from different source material or with different DE might modulate the gut microbiome in a certain way, and thereby, exert distinct immune-modulatory effects *in vivo*.

We hypothesize the DE of pectin to affect the microbiota composition and thereby the levels of metabolism and immune modulation. Therefore, this study aimed to investigate the effects of two well-defined and commercially available pectins from apple and citrus with different DE (HMP and LMP, respectively) on intestinal microbiota composition and metabolome, as well as on humoral and gastrointestinal immune responses in mice.

2. Material and methods

2.1. Diets

Two commercial pectins were provided by Herbstreith & Fox GmbH & Co. KG (Neuenbürg, Germany): Apple-derived pectin Herbapekt SF 50-LV and citrus-derived pectin Classic CU901 (Fig. 1A).

Structural characterization of the two pectins was already published (Bender et al., 2023). The thereby used abbreviations for Herbapekt SF 50-LV and Classic CU901 are AP2 and CP2, respectively. A comparatively high portion of galacturonic acid (52–61 mol%) was determined for both pectins, indicating a high amount of homogalacturonan. The neutral side chains only make up a small proportion of the total structure of both pectins and are mainly composed of linear type I arabinogalactans, while the mainly linear arabinans are only slightly represented in Herbapekt SF 50-LV and Classic CU901 (arabinose content 4.3 mol% and 3.8 mol%, respectively). Additionally, the occurrence of xylogalacturonan was proven for Herbapekt SF 50-LV, which has already been shown for apple-derived pectins (Zandleven, Beldman, Bosveld, Schols, & Voragen, 2006). Both pectins are of comparatively low molecular weight, MW of 38 to 47 kDa. The DE of Herbapekt SF 50-LV is 57.0 % so that the pectin can be assigned to HMP while Classic CU901 is a LMP with a DE of 4.4 % (Bender et al., 2023).

Food pellets for mouse experiments that contained either cellulose (20 %), HMP or LMP (5 % and 15 %, respectively, and adjusted to 20 % by adding cellulose) were prepared by ssniff Spezialdiäten GmbH (Soest, Germany).

2.2. Mouse feeding

Female, 6–8 weeks old CBA/J mice (Charles River Deutschland GmbH) were housed under specific pathogen-free conditions with free access to water and food at the specific pathogen free (SPF) animal facility of the Paul-Ehrlich-Institut (Langen, Germany). The mice were randomly assigned to the different experimental groups. All animal experiments were performed in compliance with the German animal protection law (granting authority: RP Darmstadt, Germany, Approval number: F107/2003). The mice (n = 8/group; 2 mice/cage), were fed a control diet containing 20 % cellulose for 14 days, followed by 14 days feeding of the respective pectin or control diet (Fig. 1B). Body weight, health status and food consumption were monitored every 2–3 days. All experiments were carried out in compliance with the ARRIVE guidelines and regulations.

2.3. Feces sample collection

Feces samples of CBA/J mice were freshly collected per cage at day −1, 2, 7, and 13 for the analysis of non-fermented carbohydrates. Feces samples collected at day 0, 3, 6, 9, 12, and 14 were subjected to fecal microbiome analysis (Fig. 1B). Furthermore, feces samples of day 14 were used for metabolomic analyses. The fecal samples were collected, immediately frozen and stored at −80 °C until further analysis.

2.4. Measurement of antibody levels

Antibody levels were measured in the serum of the mice, taken after 14 days of feeding of pectin or control diet using ELISA kits according to the manufacturer's recommendation, either for total IgG (Cat. 88-50400), total IgA (Cat. 88-50450), or total IgE (Cat. 88-50460) (ThermoFisher Scientific, Darmstadt, Germany). Levels of total IgA were additionally analyzed in feces samples taken at day 12. Fecal pellets were homogenized in 1× protease inhibitor (Merck KGaA, Darmstadt, Germany) and protein content was adjusted to 1 mg/mL. Total IgA was determined by ELISA following the manufacturer's instruction. Optical density was measured at 450 nm by SpectraMAX Plus 340 (Molecular Devices, Germany).

2.5. Histology analysis

Length of intestinal sections was determined, and longitudinal sections of small intestinal tissue (approximately 2 cm) were taken from the jejunum (9.5 cm distal to the duodenum) of CBA/J mice. The tissue sections were fixed in 4 % formalin and embedded in paraffin. Sections of 5 µm thickness were prepared using microtome (Microm HM355S, Thermo Scientific) and stained with hematoxylin and eosin (H&E) for morphologic analysis.

2.6. Flow cytometry

The lamina propria from small intestine of CBA/J mice was enzymatically dissociated following an adapted protocol from Weigmann et al. as described in the supplemental material (Weigmann et al., 2007). Single cell suspensions of lamina propria cells underwent Fc block with anti-CD16/32 (eBioscience, Frankfurt am Main, Germany) followed by staining with antibodies specific for extracellular molecules, viability dye (ThermoFisher Scientific) and nuclear staining (true-nuclear, BioLegend, Koblenz, Germany). The target cells were stained with selected antibodies (targeting CD45, CD11c, CD11b, MHCII, CD64, CD117, Ly6G, SiglecF, CD3, CD4, CD88, CD19, CD25, FoxP3, all BioLegend), based on several gating strategies (Fig. S1). Data were acquired using a FACS Symphony (BD) and analyzed *via* FlowJo (version 10, BD).

2.7. Separation and analysis of non-fermented oligo- and polysaccharides

For the analysis of non-fermented oligo- and polysaccharides, fecal samples were suspended in ethanol (80 %; v/v) and incubated for 4 h

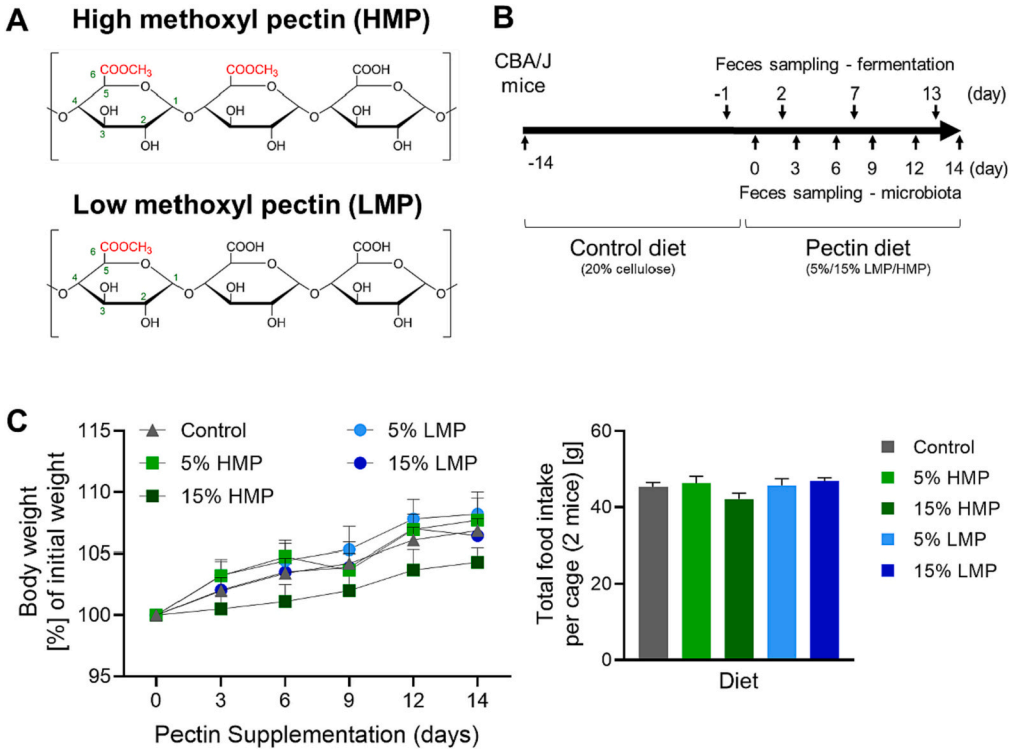

Fig. 1. Schedule of diet supplementation with pectin and effect on body weight of CBA/J mice. A) Scheme of the chemical structure of high methoxyl pectin (HMP; upper panel) and low methoxyl pectin (LMP; lower panel) with esterification at C_6 of α-(1,4)-GalA, B) Experimental design of the pectin intervention in CBA/J mice; control diet: Cellulose 20 %. C) Development of body weight over the feeding period of 14 days; n = 7–8/group.

under continuous shaking. After centrifugation (4696 ×*g*, 10 min), the solids were washed several times using 80 % (v/v) pure ethanol. Residual ethanol was evaporated and the dried polysaccharide-containing residues were analyzed for their monosaccharide composition (see Supplementary). Oligosaccharides were detected by evaporation of supernatants and dissolving in water, followed by analysis using high performance anion exchange chromatography with pulsed amperometric and mass spectrometric detection (HPAEC-PAD/MS). Evaporation residues were dissolved in acetonitrile/water (50/50; v/v) when ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS^2) analysis was performed (see Supplemental Material and Methods section).

2.8. 16S-rRNA sequence analysis of the microbiome

Bacterial DNA was extracted from feces using the NucleoSpin DNA Stool Kit (Macherey-Nagel, Düren, Germany) and quantified using the Invitrogen Qubit dsDNA HS assay on a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, USA). Samples were adjusted to a final DNA concentration of 1 ng/μL. The sequencing library for V4 16S-rRNA amplicon high throughput sequencing was prepared and paired-end sequencing was performed on an Illumina MiSeq benchtop sequencer (Illumina Inc., San Diego, USA) using MiSeq Reagent Kit v3 and PhiX Control v3 (both Illumina) as described previously (Bender et al., 2023). The PCR was carried out in final volumes of 25 μL, containing 1 μL template DNA, 1.25 μL of each primer (10 μM), and 12.5 μL of NEBNext Ultra II Q5 Mastermix (New England Biolabs GmbH). The volume of template DNA in samples lower than a concentration of 1 ng/μL was increased up to 5 μL. The PCR was carried out as follows: 30 s initial denaturation at 98 °C, followed by 25 cycles of 10 s at 98 °C, 30 s at 55 °C and 20 s at 72 °C, and a final elongation of 2 min at 72 °C.

Processing of the high throughput sequencing data was performed using the *R software* in *RStudio* (RStudio Inc., Boston, USA) (R Core Team, 2014). Demultiplexed fastq sequences were processed using *DADA2* (v.1.22.0) following the *DADA2* pipeline (Callahan et al., 2016) yielding amplicon sequencing variants (ASVs) as described previously (Bender et al., 2023). ASVs were taxonomically assigned using the SILVA138 database (Quast et al., 2013). The data was further processed and analyzed using the packages *phyloseq* (v.1.38.0) (McMurdie & Holmes, 2013) and *ggplot2* (v.3.3.6) (Wickham, 2016). ASVs assigned to chloroplasts or mitochondria were omitted.

2.9. Short-chain fatty acid and bile acid analyses

Feces samples were lyophilized in a LyoQest −85 lyophilizer (Telstar, Barcelona, Spain) until completely dried (20 h at −110 °C, 0.001 mbar). Then, the lyophilizate from each individual sample was homogenized in 1 mL of ultrapure water using 50 mg of 700–1000 μm (mean diameter) glass beads at 50 Hz for 2 min in a Tissue Disruption System (QIAGEN, Hilden, Germany), and 3 repeated cycles were performed with a 1 min rest time on ice between each. These homogenates were aliquoted and stored at −80 °C until the day of sample preparation, when they were all slowly thawed on ice.

The levels of seven short-chain fatty acids (SCFAs), comprising acetic, propionic, butyric, isobutyric, valeric, isovaleric, and 2-methyl-butyric acids were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS^2) with isotope labeling internal standards. Analytes were derivatized with dansylhydrazine (DnsHz) using an optimized and validated methodology based on Zhao and Li (Zhao & Li, 2018). Feces homogenates, spiked with labeled internal standards, were derivatized by the sequential addition of 20 μL of DnsHz, 20 μL of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), and 20 μL of 1-hydroxy-7-azabenzotriazole (HOAT). Upon shaking (22 °C, 90 min), samples were added 20 μL of copper chloride ($CuCl_2$) to quench the labeling reagents and were incubated at 40 °C for 30 min. The mixture was then centrifuged (16,100 ×*g*, 10 min, 4 °C) and 50 μL of the supernatant were taken in a vial with insert for further analysis. Lastly, vials were centrifuged at 2000 ×*g* for 10 min at 4 °C (Heraeus Megafuge 1.0R, Hanau, Germany). Samples were measured using an Agilent 1260 Infinity II LC system coupled to an Agilent 6470 triple quadrupole MS (QqQ-MS) detector (Agilent Technologies, Santa Clara, California, United States) in positive electrospray ionization (ESI) mode.

BAs were analyzed using a LC-MS^2-method for the detection of twenty-five BA. First, 950 μL of a feces homogenate (8–21 mg/mL in ultrapure water) were transferred to an Eppendorf tube. Subsequently, 1045 μL of acetonitrile:2-propanol (6:5, v/v) containing synthetic standards (3,7,12 Dehydrocholic acid (3,7,12-DHCA); 3,7,12 Glycodehydrocholic acid (3,7,12-GDHCA); and 3,7,12 Taurodehydrocholic acid (3,7,12-TDHCA)) (Steraloids Inc., New-port, Rhode Island, United States) at a concentration of 9.55 mg/L were added. Next, samples were centrifuged at 16100 ×*g* for 20 min at 4 °C in a 5415R Eppendorf centrifuge (Hamburg, Germany). 700 μL of this supernatant were further filtered through nylon membrane, 0.45 μm pore size micro-centrifugal filters (Costar, Washington D.C., United States) at 16100 ×*g* for 15 min at 4 °C. 50 μL of the resulting filtrate were taken in a vial with insert, diluted in 200 μL of ultrapure water:acetonitrile:2-propanol (10,6:5, v/v/v) with 0,625 mg/L of deuterated standards Glycocholic Acid-d4 (GCA-d4), Deoxycholic Acid-d4 (DCA-d4), Chenodeoxycholic Acid-d4 (CDCA-d4), Cholic Acid-d4 (CA-d4), and Ursodeoxycholic Acid-d4 (UDCA-d4) (Cayman Chemical, Ann Abor, Michigan, United States) and centrifuged as previously described. Samples were measured following previously published methodology using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6460 QqQ-MS detector (Agilent Technologies, Santa Clara, California, United States) in negative ESI mode (Sarafian et al., 2015).

Data were integrated using MassHunter Quantitative Analysis B.10.0 software (Agilent Technologies). SCFA quantification and BA semi-quantification was performed using internal standard calibration. The calibration curves were created using the peak area ratio of the sample analytes (SCFA or BA) and their respective labeled analogs *versus* the corresponding concentration of calibration standards.

2.10. Statistical analysis

The results are represented as means ± standard error of the mean (SEM) and the data were statistically evaluated by either Mann-Whitney *U* test or Kruskal-Wallis test ($\alpha = 0.05$). The statistical software was Graph Pad Prism v10.0.0. Additionally, for SCFAs and BAs, supervised multivariate analysis (MVA) using partial least squares-discriminant analysis (PLS-DA) and orthogonal (O)PLS-DA was performed using SIMCA P + 17.0 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). PLS-DA models were used to corroborate separation between groups and OPLS-DA models were used to obtain the significant metabolites in each comparison. All models are provided with their quality parameters R^2, which represents the percentage of the variability of the samples that the model can explain; and Q^2, which represents the capacity of the model to predict the classification of new samples. The statistical significance was set at an absolute value of correlation p-value ($|p\text{-corr}| \geq 0.5$ (meaning a correlation of at least 50 % with one group), a Variable Importance in the Projection (VIP) score >1, and a significant Jackknife confidence interval (value >0). Graphics were performed using GraphPad Prism v10.0.0 (San Diego, CA, USA).

The processed V4 16S-rRNA amplicon high throughput sequencing data was statistically analyzed in *RStudio* using the packages phyloseq (v.138.0) and ggplot2 (v.3.3.6) as described previously (Bender et al., 2023). Microbial richness was estimated using the Chao1 estimator and alpha diversity was calculated using the inverse Simpson index by using the estimate richness function, respectively (Chao, 1984; Simpson, 1949). Linear mixed models for each single intervention were calculated using *lme4* (Bates, Mächler, Bolker, & Walker, 2015) and subsequently subjected to ANOVA analysis, while the collection day was used as fixed effect and cages (n = 2 mice) as random effect. P-values below 0.001

were reported as significant. Beta diversity was estimated using the Jensen-Shannon divergence (JSD) and the ordination was visualized by presenting the data in a biplot based on principal coordinate analysis (PCoA). Biplot arrows indicate up to 20 ASVs driving separation between the samples ("loadings"), arrows are labeled with the taxonomy assigned to the respective ASV. Significant differences in beta diversity were tested by PERMANOVA analysis using the *adonis2* function implemented in *vegan* (Oksanen et al., 2018) with 9999 permutations, while R^2 and p-values are given in the results. Differential abundance analysis of ASVs was performed using *ALDEx2* (Fernandes et al., 2014; Fernandes, Macklaim, Linn, Reid, & Gloor, 2013; Gloor, Macklaim, & Fernandes, 2016) with 128 Dirichlet Monte-Carlo simulations and a generalized linear model, using collection day, intervention and cages as variables. Significant differences were reported when the p-values adjusted by the Benjamini-Hochberg method were below 0.05. The resulting graphs were finalized in *Inkscape* (v.1.2).

3. Results

3.1. Supplementation of the diet with pectins does not affect body weight gain and food intake

To compare the effect of dietary supplementation using two structurally different types of pectin, CBA/J mice were initially fed a control diet of 20 % cellulose for 14 days, followed by 14 days of pectin supplemented diet containing either 5 % or 15 % of either HMP or LMP, respectively (Fig. 1A, B). Monitoring of the body weight gain of the individual mice and food intake measured per cage during the pectin feeding period revealed an increase in the body weight in all feeding groups over time (Fig. 1C). However, mice fed a 15 % HMP diet over two weeks showed a slightly reduced total body weight gain (around 1 g per mouse) compared to the control group (around 1.4 g per mouse) (not shown). The food consumption measured per cage (n = 2 mice) showed a similar food intake of around 45 g in all five feeding groups over 14 days (Fig. 1C). The data indicated that the type of pectin did not affect body weight gain and food consumption of the mice.

3.2. LMP and HMP are fermented differently by intestinal microbiota

To examine whether HMP and LMP are fermented differently in the gut, the levels of pectin-derived oligosaccharides were determined in the feces samples of the mice at different time points. HPAEC chromatograms of the ethanol-soluble carbohydrates of fecal samples are shown in Fig. 2A. After diet supplementation with 5 % LMP no pectin-derived (pectic) oligosaccharides were detectable, suggesting an almost complete metabolization of the oligomeric pectin structures released during fermentation. In contrast, GalA and its dimer were detected at low intensities after 15 % LMP feeding, thus suggesting a concentration-

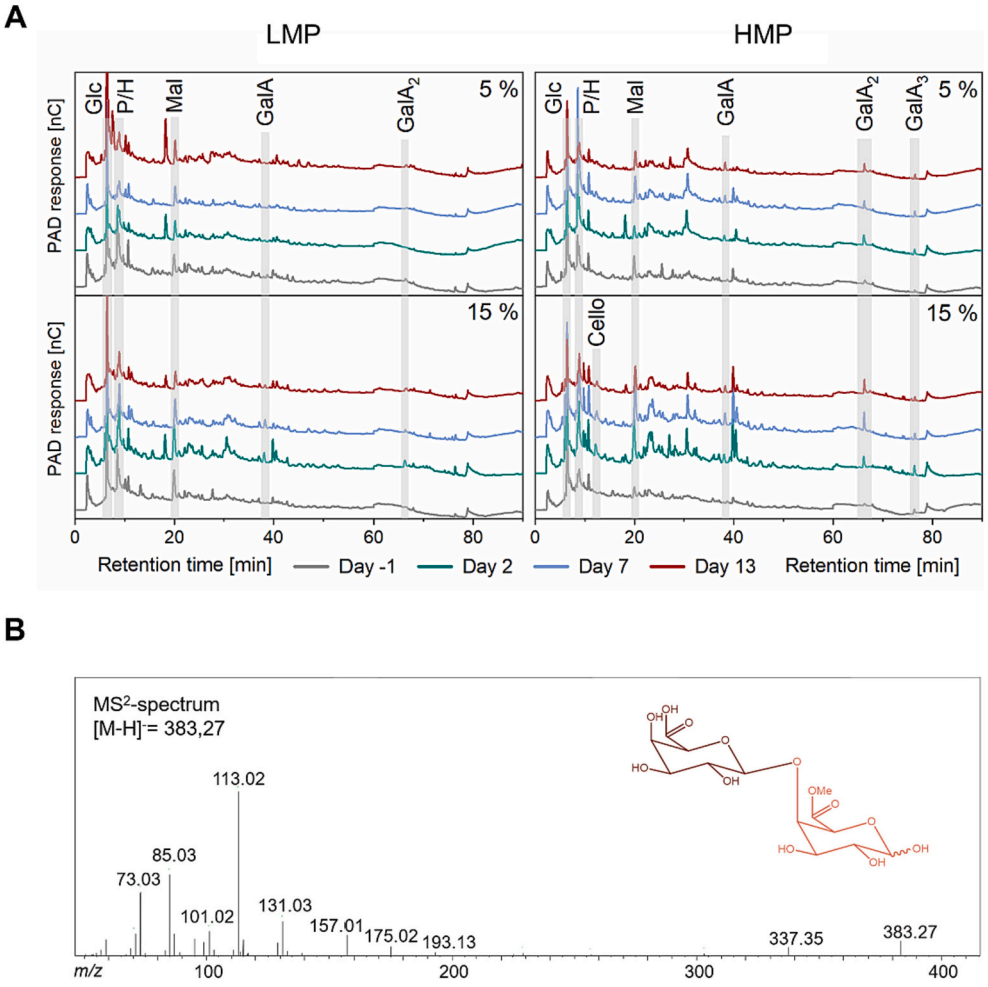

Fig. 2. Analysis of non-fermented oligosaccharides and MS^2 spectrum of GalA with an ester bound methyl group in feces samples of pectin fed CBA/J mice. A) High-performance anion-exchange chromatography (HPAEC) coupled with a pulsed amperometric detector (PAD)-derived chromatograms of ethanol-soluble fractions of mouse feces before and after feeding of either 5 % or 15 % of LMP or HMP for 14 days. Identification of fermentation products at different time points of the feeding is depicted: Cello: Cellobiose, GalA: Galacturonic acid, Glc: Glucose, H: Hexose, Mal: Maltose, P: Pentose. B) UHPLC-MS^2 based analysis with normalized collision-energy of 35 and the resulting fragmentation pattern. This structure was identified after pectin feeding of 15 % LMP, and 5 %/15 % HMP with higher intensities.

dependent fermentation process of LMP. With a 5 % HMP supplemented diet, GalA as well as its dimer and trimer were identified in the fecal samples, which pointed to an incomplete and less efficient fermentation of HMP, compared to LMP. The same pectic carbohydrates were detected with higher intensities when mice were fed a diet supplemented with 15 % HMP, showing a similar concentration-dependent effect for HMP.

By using UHPLC-MS^2 analysis an additional dimer of GalA methyl esterification was identified in the fecal samples of all mice except those that were fed a diet supplemented with 5 % LMP (Fig. 2B). This also suggested slower fermentation of HMP compared to LMP which could be supported by analysis of monosaccharide compositions of ethanol-insoluble fractions (Table S1).

In CBA/J mice receiving a 5 % LMP diet, pectin-specific monosaccharides were not found in any fraction of the feces, suggesting an extensive fermentation of polymeric pectins (Table S1). In contrast, in mice receiving 15 % LMP diet, the polymeric fraction of the murine fecal samples contained GalA with a proportion of 50.1–52.4 mol% (based on all monosaccharides that were detected in quantifiable amounts), confirming a concentration-dependent incomplete fermentation of LMP. For HMP-based diet (5 %) GalA-containing polysaccharides were detected (GalA proportions 21.4–25.2 mol%) suggesting a slower fermentation of HMP as compared to LMP in the 5 % pectin groups, which was less prominent in the 15 % pectin groups.

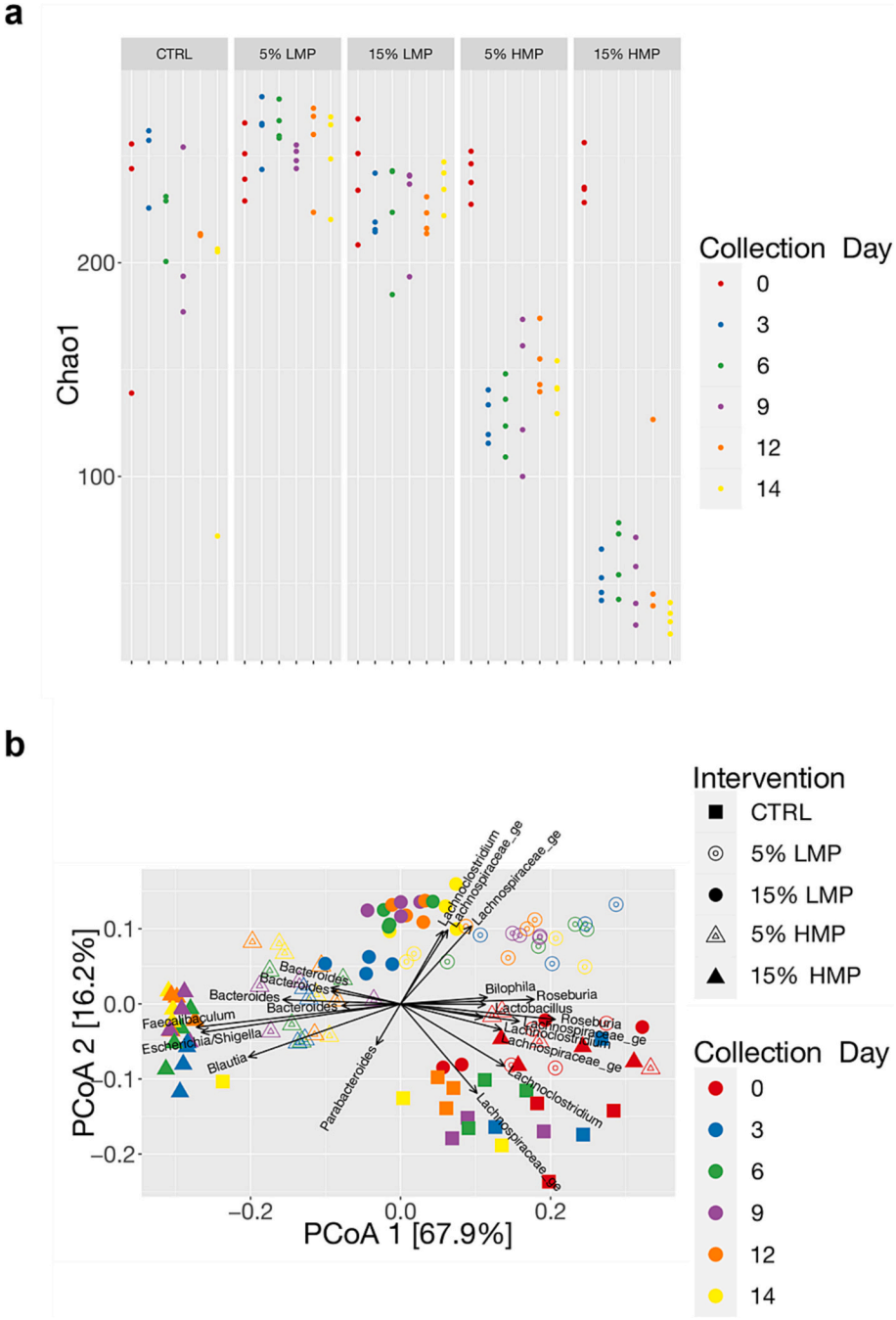

Fig. 3. Effect of different pectin diets on the fecal microbial diversity in CBA/J mice. A) Richness calculated using the Chao 1 estimator and B) beta diversity calculated as Jensen-Shannon divergence (JSD), the ordination was visualized by presenting the data in a biplot based on PCoA.

3.3. Supplementation of the diet with HMP rather than with LMP changes the composition and diversity of the gut microbiota

To determine whether the observed differences in pectin fermentability influence the bacteria composition in the gut, 16S-rRNA sequence analysis of the microbiota was performed using fecal samples at distinct time points. The microbial richness, calculated as Chao 1 index, significantly ($p < 0.001$) decreased in both groups fed a diet supplemented with HMP, while the richness was not significantly affected in the control group nor in either of the LMP-fed groups (Fig. 3A). In addition, the alpha diversity calculated as inverse Simpson index, was significantly ($p < 0.001$) reduced when the mice were fed a HMP supplemented diet (Fig. S2). The beta diversity was analyzed as PCoA (Fig. 3B). The results indicated that the majority of the baseline samples collected at day 0 cluster separately from all samples collected on day 3 and later. In addition, samples collected on day 3 and later can be distinguished by their intervention (feeding group) as they form separate clusters. Statistical testing (PERMANOVA) of the interventions, the sampling time and the cages (n = 2 mice) as independent variables yielded a strong influence of the interventions ($R^2 = 0.50$; $p = 0.001$) followed by the collection day ($R^2 = 0.19$; $p = 0.0001$), but not of the cages ($R^2 = 0.015$; $p > 0.05$), which is also reflected by the PCoA.

The mean relative abundances of amplicon sequence variants (ASVs) that have been assigned to the most abundant genera showed, that especially HMP led to a modified fecal microbiota compared to the group being fed a control diet (Fig. 4). In both HMP groups, a highly pronounced and significant increase of ASVs assigned to *Bacteroides* could be observed. In the case of 5 % HMP supplementation, next to an increase of *Bacteroides*, a significant increase in some members of the family *Lachnospiraceae* was detected, whereas a decrease in the abundance of members of *Muribaculaceae* was particularly evident. In the case of 15 % HMP diet, in addition to the increase of *Bacteroides*, a significant increase in members of the *Escherichia/Shigella*, *Olsenella*, *Paludicola*, *Enterococcus* and *Faecalibaculum* was observed. Similar to 5 % HMP feeding, a decrease of members of *Muribaculaceae* could be detected whereas in contrast to the feeding with 5 % HMP several members of the *Lachnospiraceae* also significantly decreased.

3.4. Fecal metabolome is differentially modulated by HMP and LMP

Next, to explore the influence of pectin on intestinal metabolome, targeted metabolomics for SCFAs and BAs was performed in feces samples collected at day 14. In total, 7 SCFAs and 15 BAs were detected and quantified (Table S2). Among them, primary BAs were detected, including cholic acid (CA), taurocholic acid (TCA), chenodeoxycholic acid (CDCA), tauro-α muricholic acid (TαMCA), and β-muricholic acid (β-MCA); but preferentially secondary BAs, including deoxycholic acid (DCA), lithocholic acid (LCA), isolithocholic acid (iLCA), ursodeoxycholic acid (UDCA), tauro-ursodeoxycholic acid (TUDCA), 5β-cholanic acid-3β, 12α-diol (3β-DCA), 3α-hydroxy-7 ketolithocholic acid (7-KLCA), 5-cholenic acid-3β-ol (5-cholenic), tauro-ω muricholic acid (TωMCA), and murocholic acid (MDCA) (not shown).

Multi variant analysis (MVA) considering both SCFAs and BAs datasets was applied between the groups that were fed a pectin supplemented diet containing 5 % or 15 % of either HMP or LMP (Fig. 5A). The PLS-DA model showed that the biggest differences occurred between mice that were fed with 15 % pectin, as they appeared at the edges of the model, while the groups that received 5 % pectin were in the center without being separated from each other. These findings suggest that the effect of pectin in the fecal metabolome is not only treatment- but also concentration-dependent.

To corroborate this, discriminant multivariate models, comparing the pectin concentration and type and dose separately were performed. In the first comparison, resulting PLS-DA models explained >75 % of the samples' classification in the 5 % or 15 % group of either HMP and LMP with moderate and good prediction scores ($Q^2 = 0.396$ and 0.836, respectively) (Fig. S3A, B). In addition, the comparisons of each pectin with their specific DE to the control group can be found in Fig. S3C–F). However, as expected, the highest variation was obtained between the groups that were fed high amounts of either HMP or LMP (PLS-DA and OPLS-DA: $R^2 > 0.9$ and $Q^2 > 0.75$), confirming that these groups had a specific metabolomic fingerprint that differentiated them from each other (Fig. 5B, C). Therefore, further analysis mainly focused on both groups supplemented with 15 % pectin.

To determine which metabolites were accountable for the difference between 15 % HMP and 15 % LMP groups, SCFAs and BAs with a correlation p-value $|p\text{-corr}| > 0.5$, a VIP score > 1, and a significant Jackknife confidence interval (value >0) from the OPLS-DA model were

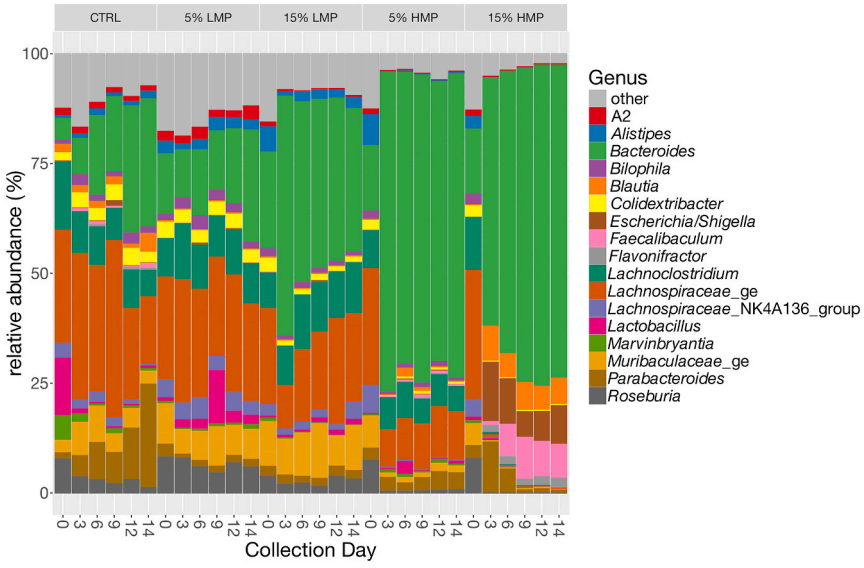

Fig. 4. Effect of different pectin diets on the composition of the fecal microbiota in CBA/J mice. Mean relative abundances of amplicon sequence variants (ASV) that have been assigned to the 17 most abundant genera of the bacterial microbiota.

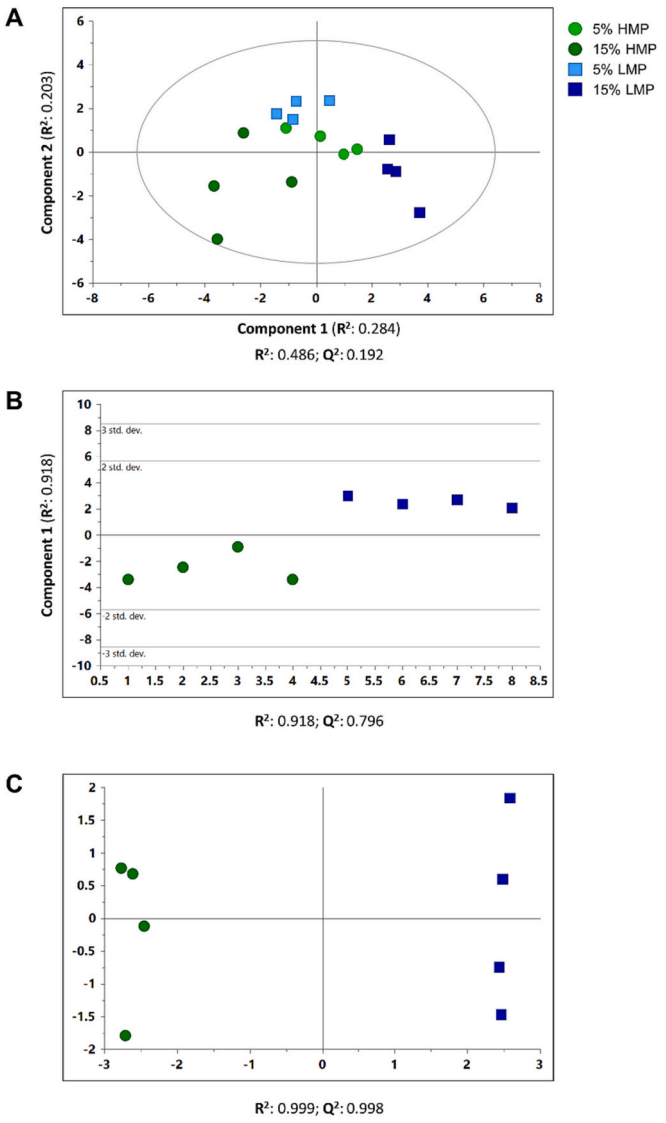

Fig. 5. Multivariate statistical analysis of the fecal metabolomic profile for bile acids and short-chain fatty acids. Supervised multivariate models for different comparisons after 14 days of pectin feeding. A) PLS-DA multivariate model comparing all pectin feeding groups. B) PLS-DA and C) Cross-validated OPLS-DA multivariate models comparing 15 % HMP and 15 % LMP groups. n = 4/group. All models are provided with their quality parameters R^2 (percentage of the variability of the samples that the model is able to explain) and Q^2 (capacity of the model to predict the classification of new samples). All multivariate models were built with univariate scaling (UV) and without any transformation of the data.

selected (Fig. 5C). A total of 13 significant metabolites was obtained, including SCFAs (acetic and isovaleric acids), primary BAs (TCA, TαMCA), and secondary BAs (MDCA, TωMCA, 3β-DCA, TUDCA, iLCA, LCA, DCA, 7-KLCA, UDCA) (Fig. 6). Interestingly, most of these metabolites were found to be increased in the 15 % LMP group compared to the 15 % HMP group, except for 7-KLCA and UDCA, which were decreased.

The only SCFA which was significantly elevated compared with the control group was 2-methyl butyric acid in response to HMP. A significant increase in BA production in comparison with the control was also detected for MDCA, ILCA, LCA (in response to LMP), and 7-KLCA and UDCA (in response to HMP). Remarkably, CA was substantially reduced in both pectin groups in comparison with the control (Fig. 6).

In summary, after 14 days of treatment with 5 % or 15 % of either HMP or LMP, the results of metabolome analysis showed a modulation of the fecal metabolome that is both treatment- and dose-specific. HMP 15 % modulates humoral and fecal IgA immune responses.

3.5. Pectins affect antibody production in mice

Next, the immune-modulatory capacities of the pectins were investigated. Analysis of the antibody levels in the serum of the mice by ELISA showed a significant increase in the total IgG level when mice were fed a diet enriched with 15 % HMP but not with 15 % LMP (Fig. 7A). However, total IgE levels were not significantly affected by the different diets (Fig. 7B). Analysis of the ratio of total IgG to total IgE levels showed a significant increase only when the mice were fed a diet enriched with 15 % HMP (Fig. 7C). In addition, the data revealed a significant increase of total IgA levels (6-times enhanced compared to the control group) when mice were fed a 15 % HMP supplemented diet (Fig. 7D). In line with this, IgA levels in fecal samples levels were also significantly higher with both pectins (Fig. 7E).

In summary, a strong modulation of antibody responses could be observed, particularly after diet supplementation with HMP, which was characterized by a strong induction of total IgG and IgA and increased total IgG/IgE ratios.

3.6. High concentrations of HMP and LMP exert distinct effects on intestine physiology and infiltrating cells

To examine, whether diet supplementation with different pectins induced further physiological alterations in the intestinal tract of CBA/J mice, the morphology of the intestine was analyzed. The lengths of three intestinal sections (small intestine, large intestine, and caecum) were measured after 14 days of supplementation with pectin (Fig. S4). Feeding HMP 15 % resulted in a slight increase (1.06-fold, 6.4 %) in the length of the small intestine. In contrast, after HMP 15 % feeding, the large intestine and caecum were significantly enlarged, 1.2-fold and 1.7-fold, respectively. In addition, treatment with LMP 15 % resulted in a 1.08-fold increase in the length of the large intestine and with HMP 5 % a 1.3-fold increase in the length of the caecum.

Although the histological analysis of the jejunum (distal part of the small intestine) showed only scattered inflammatory cells (Fig. S5), the characterization of infiltrating cells in the lamina propria by flow cytometry revealed strong immune-modulatory effects induced by the two different pectins (Fig. 8). Results showed a significant decrease of T cells after supplementation with 15 % LMP. Additionally, whereas the frequency of T helper (Th) cells significantly enhanced by 15 % HMP, it also significantly decreased the number of regulatory T cell (T_{regs}). The number of B cells was slightly enhanced in all pectin feeding groups. In contrast, a strong and significant reduction of conventional dendritic cells (cDCs), macrophages, eosinophils, and mast cells was achieved by supplementation with 15 % HMP, but not LMP.

In summary, dietary supplementation with HMP - but not LMP - was associated with a reduced infiltration of antigen-presenting cells and inflammatory cells in the intestinal mucosa.

4. Discussion

The dietary fiber pectin has been described to contribute to gut microbiota homeostasis and exerting health-promoting effects (Hasan & Yang, 2019; Schwartz et al., 1982). Previous studies have shown that the utilization of pectins by intestinal bacteria depends on their structural characteristics (Bender et al., 2023; Beukema et al., 2022). However, it is not known whether certain pectins are capable of modulating immune responses more efficiently than others (Firrman et al., 2022; Pascale et al., 2022). Therefore, the present study aimed to compare the prebiotic and immune-modulatory properties of two structurally different pectins (HMP and LMP) in CBA/J mice.

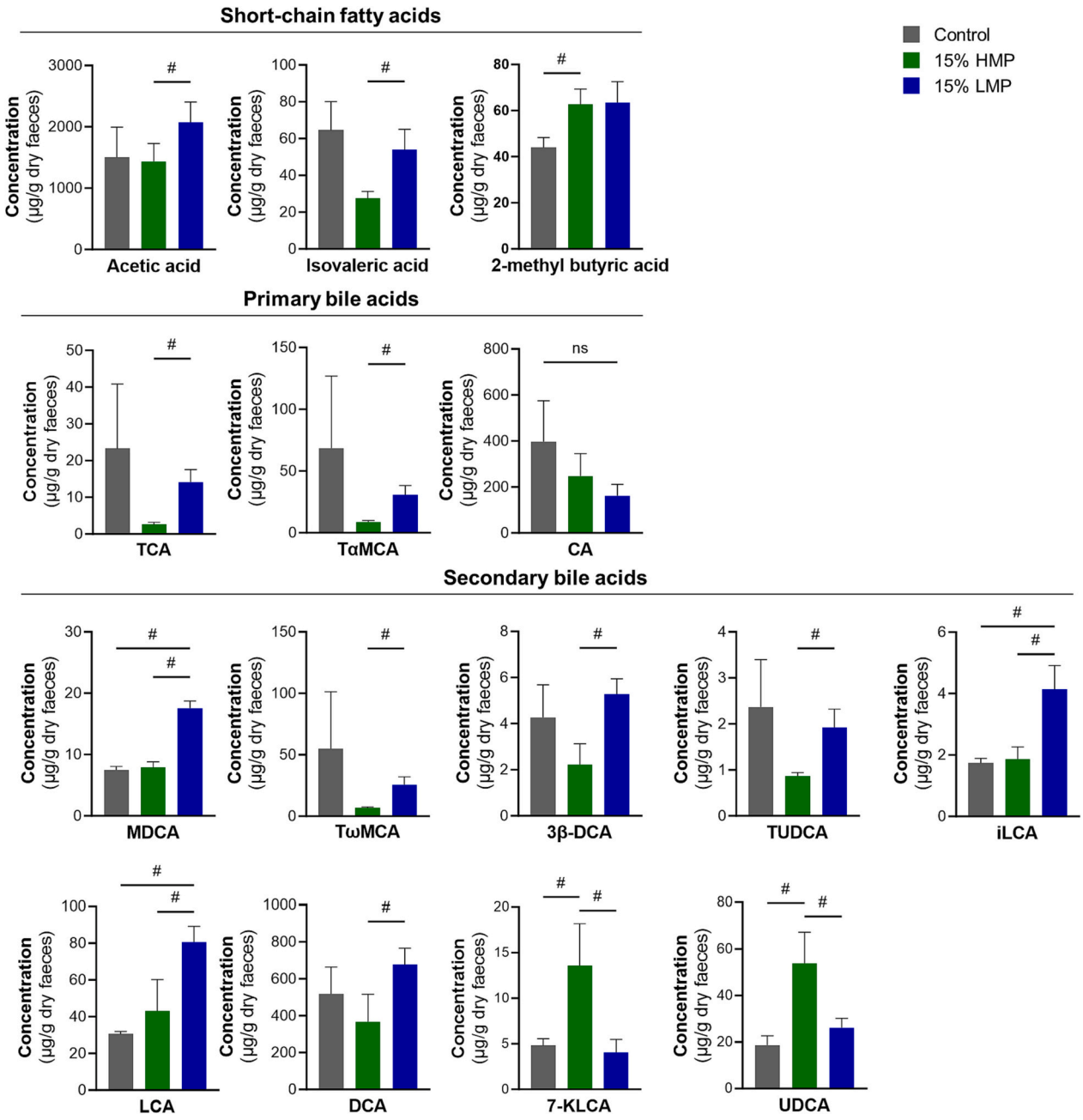

Fig. 6. Individual trajectories of the significant metabolites by multivariate analysis in the comparison between 15 % HMP, 15 % LMP, and control groups. Trajectories of the metabolites (SCFAs and primary/secondary BAs) that were found significantly altered after multivariate analysis between the two groups that were fed with a dietary supplementation of 15 % pectin (either HMP or LMP), and in their comparison with the control group (n = 3–4/group). Values are presented as the mean ± the standard error of the mean (SEM) and significance is shown by # (|p-corr| >0.5, VIP >1, and a Jackknife confidence interval >0).

Our results showed that neither HMP nor LMP significantly affected the food intake or body weight gain of the mice. Furthermore, no disease-related symptoms or inflammatory processes could be observed by supplementation either with pectin or cellulose, indicating good tolerance of the diet by the mice. Although dietary fibers such as pectins can directly interact with intestinal cells and mucosal immune cells (Beukema, Faas, & Vos, 2020; Chen et al., 2006; Prado et al., 2020; Sahasrabudhe et al., 2018) the predominant effect of pectin is suggested to be induced by altering the composition of the gut microbiota.

Results of the present study indicated that the impact of pectin on the gut microbiota composition of mice is likely attributed to the fermentability of structurally distinct pectins. Based on the detection of GalA and its oligomers as structural units from partly fermented pectins, a slower fermentation of HMP in comparison to LMP is suggested, which is consistent with previous findings in feeding studies with rats (Nyman & Asp, 1982; Tian et al., 2016), and by *in vitro* fermentation of human feces samples (Bender et al., 2023). Studies suggest that LMPs are preferred substrates for pectin-depolymerizing enzymes of intestinal bacteria, which may explain their faster fermentation (Dongowski & Lorenz, 1998).

However, the less efficient fermentation of HMP was inversely correlated with its effect on the intestinal microbiota. The microbial analysis of fecal samples showed a strong and dose-dependent reduction in the microbial diversity when the mice were fed an HMP supplemented diet. This loss of diversity might be due to a significant increase in ASVs assigned to *Bacteroides* (Pascale et al., 2022) which was also found in our

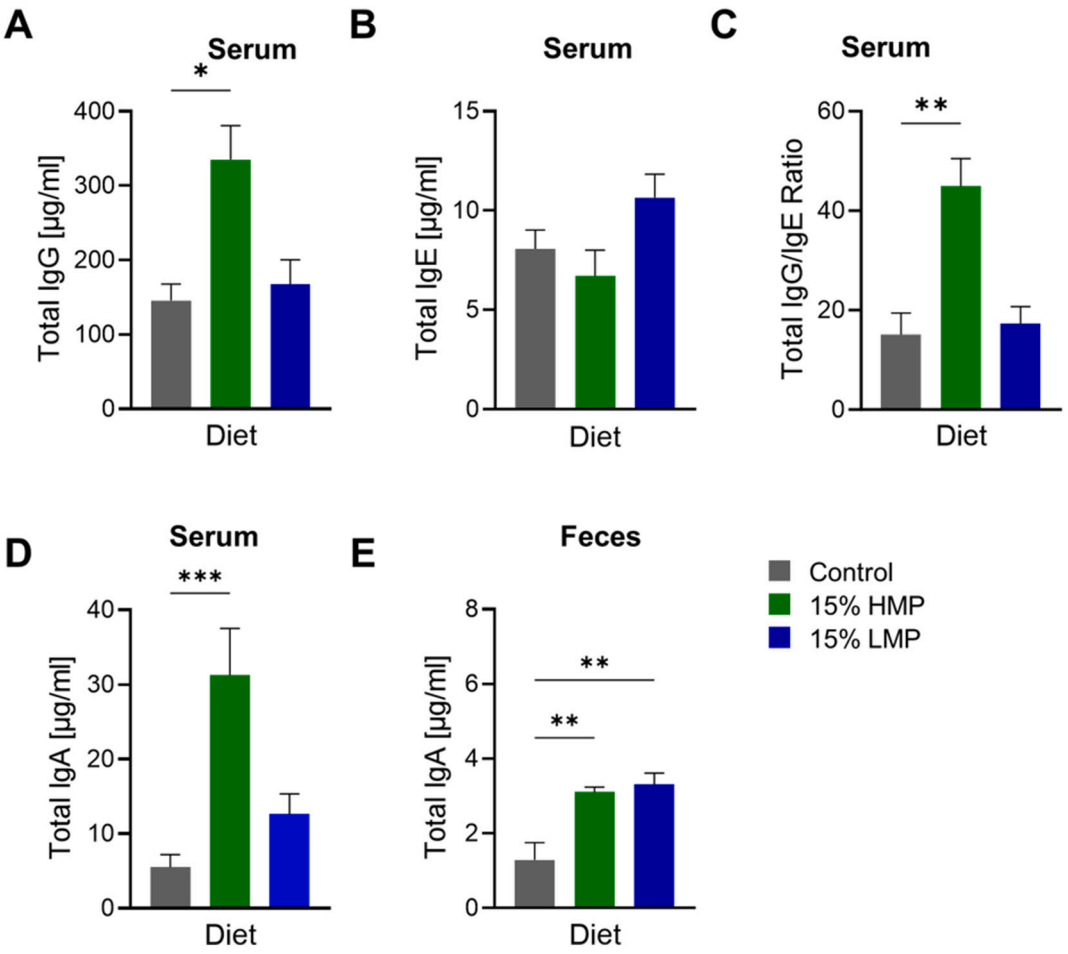

Fig. 7. Induction of antibody responses after diet supplementation with pectin in CBA/J mice. Immunoglobulin levels were evaluated in the serum for A) total IgG, B) total IgE, C) ratio of total IgG to total IgE and D) total IgA after two weeks of pectin feeding. E) Levels of total IgA in feces samples of the mice collected after 12 days; n = 7–8/group; *p < 0.05; **p < 0.01; ***p < 0.001.

study. Members of the genus *Bacteroides* are well-known to degrade pectins as they are able to respond quickly to the presence of polysaccharides (Chung et al., 2017; Martens et al., 2011; Ndeh & Gilbert, 2018). In addition, the observed highly variable response of members of *Lachnospiraceae* to pectin is consistent with the literature as varying enzymatic properties have been described for this family (Pascale et al., 2022). Moreover, a total of 40 ASVs that were assigned to *Muribaculaceae* were detected. This bacterial family was firstly described in 2019 (Lagkouvardos et al., 2019), and members of this family being *inter alia* described for their capacity to degrade complex plant cell wall glycans, *e.g.* pectin (Ormerod et al., 2016). Interestingly, especially supplementation with HMP modulated the bacterial diversity and composition, whereas LMP exerted only minor modulatory effects. One might speculate that, depending on the chemical structure, pectins can be utilized differently by bacterial strains. Furthermore, the production of different intermediate pectin breakdown products likely affects the composition and diversity of the gut microbiota.

Remarkably, feeding of two structurally different pectins exerted different effects on the humoral immune response in mice. HMP, but not LMP, elicited a substantial and significant induction of total IgG responses, and an enhanced total IgG/IgE ratio. In line with this, it has been reported that a high IgG/IgE ratio reflects an immune deviation from a Th2 (IgE) to a Th1 (IgG) immune response. In addition, several commensal bacterial taxa are suggested to evoke serum IgA responses (Wilmore et al., 2018; Zeng et al., 2016). In the present study, levels of serum and fecal IgA were increased in all pectin feeding groups compared to the control. Moreover, high IgA levels have been potentially associated with a low risk of food allergy, that is explained by IgE-blocking capacity, immune exclusion, or inhibition of IgE-mediated mast cell activation (El Ansari et al., 2022; Shamji et al., 2021).

Both, the dose (5 % *versus* 15 %), and the type of pectin (LMP *versus* HMP) induced different effects on the levels of both SCFAs and BAs in feces samples. In accordance with the literature, we found that LMP yielded higher levels of several metabolites than HMP (Alexander et al., 2023; Baxter et al., 2019; Pezzali, Shoveller, & Ellis, 2021). SCFAs, such as acetic and isovaleric acids result from the fermentation of fibers (Rios-Covian et al., 2020). Acetic acid regulates the pH level and maintains the homeostasis of the intestinal environment, nourishes beneficial microorganisms, and prevents the invasion of harmful bacteria and opportunistic pathogens (Dang et al., 2022). Butyric acid is a *Bacteroides*-derived SCFA with strong immune modulating properties (Alexander et al., 2023; Blanco-Pérez et al., 2021). However, in the present study, the only SCFA which was significantly induced in comparison to the control group was 2-methyl-butyric acid. In line with this, a diminished SCFA production was observed in pectin-treated mice in comparison to the control (Beukema et al., 2022).

Moreover, both pectins induced a different metabolic shift of bile acid production. In particular, 3 primary BAs (such as TCA, TαMCA, and CA derived from liver cells) and 9 secondary BAs (such as 7-KLCA and UDCA derived from bacteria) were affected. These observations indicate that the detected BAs are mostly microbiota-derived metabolites and include the muricholic acid family, which have recently been found to have anti-inflammatory properties (Xiao et al., 2022). Given these results, it is tempting to speculate whether certain pectin structures may

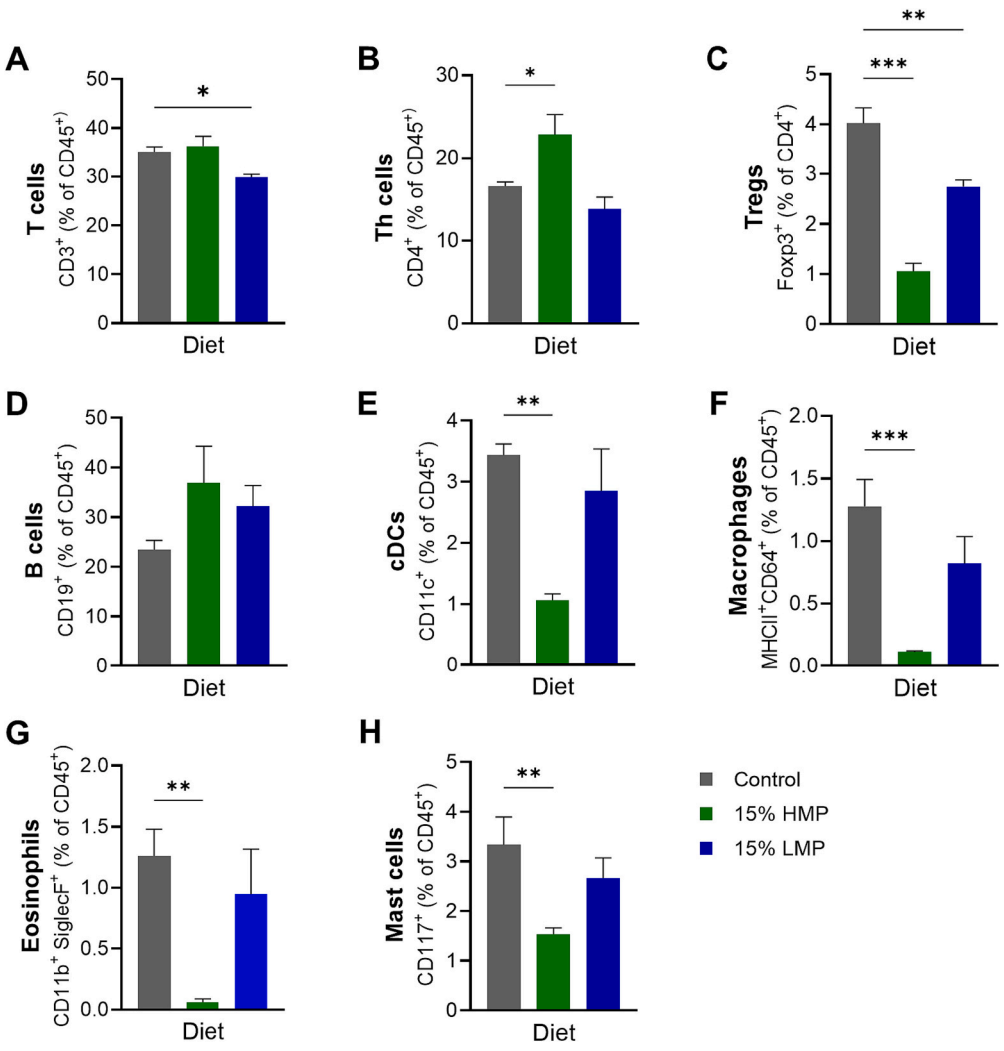

Fig. 8. Impact of pectin consumption on infiltrating immune cells in the lamina propria of CBA/J mice. Flow cytometric analysis of infiltrating immune cells in the lamina propria of the small intestine in CBA/J mice upon 14 days of diet supplementation with HMP and LMP; n = 3–4/group; *p < 0.05.

induce the production of secondary BAs by modulating the intestinal bacteria with respective metabolic activities. Currently there are almost no reports available regarding the effect of dietary fiber on BA production. Except for a recent study which reported that dietary fiber and prebiotic inulin induced CA which was associated with a type 2 inflammation (Arifuzzaman et al., 2022). These results are in agreement with the present study providing evidence that supplementation with pectin induced a substantial reduction of CA, which correlated with a reduced infiltration of inflammatory eosinophils and mast cells into the lamina propria. This data demonstrated that bacterial-derived metabolites are differentially regulated by both pectins. However, further studies are needed to explore the underlying mechanism, and to substantiate the role of each metabolite on the immunomodulatory effect.

In addition to the modulatory effects on gut microbiome and humoral immune response, the impact of structurally different pectins was investigated on the intestinal morphology as well as the infiltrating cells of the lamina propria. Supplementation of diet with HMP tended to slightly lengthen the small intestine and significantly enlarged the large intestine, as well as the caecum, whereas the effect was less prominent for LMP. This observation could be based on different fermentation processes of structurally different pectins, as HMP is mainly fermented in the proximal colon and therefore might lead to enhanced caecum size, whereas LMPs are more efficiently fermented by the microbiota in the ileum (Tian et al., 2017). A recent study showed that pectin supplementation affects intestinal Th cell subsets, MLN-derived Tregs and induce AHR activation (Beukema et al., 2022). The T cell immunogenicity was affected by both, DE and the degree of blockiness (DB) of esterified GalA, in a sense that either high DE or high DB were required, whereas low DE in combination with low DB had no effects. Remarkably, the monosaccharide composition of all investigated pectin types showed only minor differences and SCFA production was not associated with T-cell modulating effects. Therefore, we speculate that different structural properties of pectins, and combination thereof, likely induce different immunological effects, on either T cells, inflammatory responses, AHR and/or TLR2–1 activation. In line with this, feeding of HMP (15 %), in contrast to feeding of LMP (15 %), showed a significant effect on the abundance of immune cells in the gut, reduced frequency of eosinophils and mast cells, as well as macrophages and dendritic cells, which are involved in inflammatory immune responses and antigen presentation.

5. Conclusion

This study demonstrates that structurally different pectins exert distinct immune-modulatory effects in mice. The effects might be due to structure-dependent fermentation processes, with HMP being degraded less efficiently resulting in different pectin-derived intermediate products. Supplementation of diet with HMP, rather than LMP, induced

strong effects on the microbiota, shifting toward *Bacteroides* and decreasing diversity. In line with this, especially HMP seemed to affect the humoral immune response of the mice, observed as increased levels of total IgG and IgA in serum as well as fecal IgA. Similarly, a HMP supplemented diet led to significant changes of the infiltrating immune cells in the lamina propria of the small intestine, whereas LMP induced only minor effects. In summary, this study suggests that pectin with a high DE can modulate the gut microbiome more efficiently and exerted stronger immune-modulatory effects compared to LMP. However, it remains elusive whether the observed immune responses are mediated indirectly by modulation of the microbiota, or by direct interactions of pectin-derived oligosaccharides with the immune cells, and to which extend other structural features in addition to esterification contribute to this effects.

The present study has established the basis for the immunomodulatory effects of pectin under healthy conditions and has shown that dietary supplementation with pectin with certain characteristics induces different immunomodulatory effects. Consequently, a future perspective is to evaluate whether dietary supplementation with pectins with certain structural characteristics, *e.g.* the degree of esterification, can influence the development of immune diseases, such as allergic diseases.

CRediT authorship contribution statement

Hanna Steigerwald: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Frank Blanco-Pérez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Andrea Macías-Camero:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Melanie Huch:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Conceptualization. **Caroline Bender:** Writing – original draft, Visualization, Validation, Methodology, Investigation. **Stefan Schülke:** Project administration, Conceptualization. **Judith Keller:** Supervision, Methodology. **Maren Krause:** Investigation. **Coral Barbas:** Methodology. **Irene Gonzalez-Menendez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation. **Leticia Quintanilla-Martinez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Masako Toda:** Writing – review & editing, Conceptualization. **Domingo Barber:** Supervision, Funding acquisition, Conceptualization. **Sabine Kulling:** Supervision, Project administration, Funding acquisition, Conceptualization. **Mirko Bunzel:** Supervision, Project administration, Funding acquisition, Conceptualization. **Stefan Vieths:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Alma Villaseñor:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization. **Dominic Stoll:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Stephan Scheurer:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors do not have a conflict of interest. CoI statement was provided using https://declarations.elsevier.com/ or the ICMJE disclosure form. SV received honoraria as Associate Editor of JACI from AAAI, support from AAAI and EAACI and royalties from Schattauer Allergologie Handbuch Elsevier Nahrungsmittelallergien und Intoleranzen Karger Food Allergy: Molecular Basis and Clinical Practice.

Data availability

The data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. In addition, microbiota data of 16S high-throughput amplicon sequencing of the fecal samples are deposited at NCBI (BioProject accession number PRJNA936003).

Acknowledgements

The authors thank Prof. Dr. Hans-Ulrich Endreß for providing the pectins, Dr. Sebastian Soukup for providing the scheme of the chemical structures of the pectins, and Ann-Christine Junker for the graphical abstract. The present study is part of an IGF Project of the FEI which is supported *via* AiF (AiF20528) within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Research (BMWi), and is partly funded by the German Federal Ministry for Education and Research (BMBF), BMBF-ERA 01EA1901, the Ministry of Science and Innovation in Spain (PCI2018-092930), and the European program ERA HDHL — Nutrition and the Epigenome, project Dietary Intervention in Food Allergy: Microbiome, Epigenetic and Metabolomic interactions (DIFAMEM) AC18/00031. This work was also supported by ISCIII (PI19/00044), cofunded by FEDER "Investing in your future" for the thematic network and cooperative research centres ARADyAL RD16/0006/0015 and RICORS Red de Enfermedades Inflamatorias (REI) RD21 0002 0008; as well as by the grant from Ministerio de Ciencia, Innovación y Universidades cofinanced with FEDER RTI2018-095166-B-I00. AMC is supported by an FPI-CEU postdoctoral fellowship of the university CEU, Madrid, Spain.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2024.122007.

References

Alexander, C., Brauchla, M., Sanoshy, K. D., Blonquist, T. M., Maloney, G. N., Mah, E., … Swanson, K. S. (2023). Bowel habits, faecal microbiota and faecal bile acid composition of healthy adults consuming fruit pomace fibres: Two-arm, randomised, double-blinded, placebo-controlled trials. *The British Journal of Nutrition, 130*(1), 42–55. https://doi.org/10.1017/S0007114522002951

Arifuzzaman, M., Won, T. H., Li, T.-T., Yano, H., Digumarthi, S., Heras, A. F., … Artis, D. (2022). Inulin fibre promotes microbiota-derived bile acids and type 2 inflammation. *Nature, 611*(7936), 578–584. https://doi.org/10.1038/s41586-022-05380-y

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*(1). https://doi.org/10.18637/jss.v067.i01

Baxter, N. T., Schmidt, A. W., Venkataraman, A., Kim, K. S., Waldron, C., & Schmidt, T. M. (2019). Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. *MBio, 10*(1). https://doi.org/10.1128/mBio.02566-18

Bender, C., Stoll, D., Huch, M., Weinert, C., Dötsch, A., Dräger, H., … Bunzel, M. (2023). Time-dependent fermentation of different structural units of commercial pectins with intestinal bacteria. *Carbohydrate Polymers, 308*, Article 120642. https://doi.org/10.1016/j.carbpol.2023.120642

Beukema, M.[. M.]., Faas, M. M.[. M. M.]., & Vos, P.d.[. P.]. (2020). The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: Impact via gut microbiota and direct effects on immune cells. *Experimental & Molecular Medicine, 52*(9), 1364–1376. https://doi.org/10.1038/s12276-020-0449-2

Beukema, M.[. M.]., Jermendi, É., Oerlemans, M. M. P., Logtenberg, M. J., Akkerman, R., An, R., … Vos, P.d.[. P.]. (2022). The level and distribution of methyl-esters influence the impact of pectin on intestinal T cells, microbiota, and Ahr activation. *Carbohydrate Polymers, 286*, Article 119280. https://doi.org/10.1016/j.carbpol.2022.119280

Blanco-Pérez, F., Steigerwald, H., Schülke, S., Vieths, S., Toda, M., & Scheurer, S. (2021). The dietary Fiber pectin: Health benefits and potential for the treatment of allergies by modulation of gut microbiota. *Current Allergy and Asthma Reports, 21*(10), 43. https://doi.org/10.1007/s11882-021-01020-z

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition, 41*(Suppl. 1), I11–I16. https://doi.org/10.1007/s00394-002-1102-7

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). Dada2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Canteri-Schemin, M. H., Fertonani, H. C. R., Waszczynskyj, N., & Wosiacki, G. (2005). Extraction of pectin from apple pomace. *Brazilian Archives of Biology and Technology, 48*(2), 259–266. https://doi.org/10.1590/S1516-89132005000200013

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics, 11*, 265–270.
Chen, C.-H., Sheu, M.-T., Chen, T.-F., Wang, Y.-C., Hou, W.-C., Liu, D.-Z., … Liang, Y.-C. (2006). Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. *Biochemical Pharmacology, 72*(8), 1001–1009. https://doi.org/10.1016/j.bcp.2006.07.001
Chung, W. S. F., Meijerink, M., Zeuner, B., Holck, J., Louis, P., Meyer, A. S., … Duncan, S. H. (2017). Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon. *FEMS Microbiology Ecology, 93*(11). https://doi.org/10.1093/femsec/fix127
Dang, G., Wang, W., Zhong, R., Wu, W., Chen, L., & Zhang, H.[. H.]. (2022). Pectin supplement alleviates gut injury potentially through improving gut microbiota community in piglets. *Frontiers in Microbiology, 13*, Article 1069694. https://doi.org/10.3389/fmicb.2022.1069694
Dongowski, G. (1997). R. L. Whistler and J. N. BeMiller: Carbohydrate Chemistry for Food Scientists. 241 pages, numerous figures and tables. Eagan Press, St. Paul, MN, USA, 1997. Price: 114.00 US$. *Food/Nahrung, 41*(5), 318. https://doi.org/10.1002/food.19970410523
Dongowski, G., & Lorenz, A. (1998). Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human faecal flora. *Carbohydrate Research, 314*(3–4), 237–244. https://doi.org/10.1016/s0008-6215(98)00304-8
Dranca, F., & Oroian, M. (2018). Extraction, purification and characterization of pectin from alternative sources with potential technological applications. *Food Research International (Ottawa, Ont.), 113*, 327–350. https://doi.org/10.1016/j.foodres.2018.06.065
El Ansari, Y. S., Kanagaratham, C., Burton, O. T., Santos, J. V., Hollister, B.-M. A., Lewis, O. L., … Oettgen, H. C. (2022). Allergen-specific IgA antibodies block IgE-mediated activation of mast cells and basophils. *Frontiers in Immunology, 13*, Article 881655. https://doi.org/10.3389/fimmu.2022.881655
El Kaoutari, A., Armougom, F., Gordon, J. I., Raoult, D., & Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nature Reviews. Microbiology, 11*(7), 497–504. https://doi.org/10.1038/nrmicro3050
Fernandes, A. D., Macklaim, J. M., Linn, T. G., Reid, G., & Gloor, G. B. (2013). Anova-like differential expression (ALDEx) analysis for mixed population RNA-Seq. *PloS One, 8*(7), Article e67019. https://doi.org/10.1371/journal.pone.0067019
Fernandes, A. D., Reid, J. N., Macklaim, J. M., McMurrough, T. A., Edgell, D. R., & Gloor, G. B. (2014). Unifying the analysis of high-throughput sequencing datasets: Characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome, 2*, 15. https://doi.org/10.1186/2049-2618-2-15
Firrman, J., Mahalak, K., Bobokalonov, J., Liu, L., Lee, J.-J., Bittinger, K., … Lemons, J. M. S. (2022). Modulation of the gut microbiota structure and function by two structurally different lemon pectins. *Foods, 11*(23), 3877. https://doi.org/10.3390/foods11233877
Flourie, B., Vidon, N., Florent, C. H., & Bernier, J. J. (1984). Effect of pectin on jejunal glucose absorption and unstirred layer thickness in normal man. *Gut, 25*(9), 936–941. https://doi.org/10.1136/gut.25.9.936
Fukunaga, T., Sasaki, M., Araki, Y., Okamoto, T., Yasuoka, T., Tsujikawa, T., … Bamba, T. (2003). Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain fatty acid production and microbial population. *Digestion, 67*(1–2), 42–49. https://doi.org/10.1159/000069705
Gloor, G. B., Macklaim, J. M., & Fernandes, A. D. (2016). Displaying variation in large datasets: Plotting a visual summary of effect sizes. *Journal of Computational and Graphical Statistics, 25*(3), 971–979. https://doi.org/10.1080/10618600.2015.1131161
Gómez, B., Gullón, B., Remoroza, C., Schols, H. A.[. H. A.]., Parajó, J. C., & Alonso, J. L. (2014). Purification, characterization, and prebiotic properties of pectic oligosaccharides from orange peel wastes. *Journal of Agricultural and Food Chemistry, 62*(40), 9769–9782. https://doi.org/10.1021/jf503475b
Hasan, N., & Yang, H. (2019). Factors affecting the composition of the gut microbiota, and its modulation. *PeerJ, 7*, Article e7502. https://doi.org/10.7717/peerj.7502
Kiriyama, Y., & Nochi, H. (2021). Physiological role of bile acids modified by the gut microbiome. *Microorganisms, 10*(1). https://doi.org/10.3390/microorganisms10010068
Lagkouvardos, I., Lesker, T. R., Hitch, T. C. A., Gálvez, E. J. C., Smit, N., Neuhaus, K., … Clavel, T. (2019). Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome, 7*(1), 28. https://doi.org/10.1186/s40168-019-0637-2
Larsen, N., Bussolo de Souza, C., Krych, L., Barbosa Cahú, T., Wiese, M., Kot, W., … Jespersen, L. (2019). Potential of pectins to beneficially modulate the gut microbiota depends on their structural properties. *Frontiers in Microbiology, 10*, 223. https://doi.org/10.3389/fmicb.2019.00223
Liang, R.-H., Chen, J., Liu, W., Liu, C.-M., Yu, W., Yuan, M., & Zhou, X.-Q. (2012). Extraction, characterization and spontaneous gel-forming property of pectin from creeping fig (*Ficus pumila* Linn.) seeds. *Carbohydrate Polymers, 87*(1), 76–83. https://doi.org/10.1016/j.carbpol.2011.07.013
Martens, E. C., Lowe, E. C., Chiang, H., Pudlo, N. A., Wu, M., McNulty, N. P., … Gordon, J. I. (2011). Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biology, 9*(12), Article e1001221. https://doi.org/10.1371/journal.pbio.1001221
McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PloS One, 8*(4), Article e61217. https://doi.org/10.1371/journal.pone.0061217
Ndeh, D., & Gilbert, H. J. (2018). Biochemistry of complex glycan depolymerisation by the human gut microbiota. *FEMS Microbiology Reviews, 42*(2), 146–164. https://doi.org/10.1093/femsre/fuy002
Nyman, M., & Asp, N. G. (1982). Fermentation of dietary fibre components in the rat intestinal tract. *The British Journal of Nutrition, 47*(3), 357–366. https://doi.org/10.1079/BJN19820047
Oksanen, J., Simpson, L. G., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., … Weedon, J. (2018). Vegan: Community ecology package. In *Ordination methods, di765 versity analysis and other functions for community and vegetation ecologists*.
Ormerod, K. L., Wood, D. L. A., Lachner, N., Gellatly, S. L., Daly, J. N., Parsons, J. D., … Hugenholtz, P. (2016). Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. *Microbiome, 4*(1), 36. https://doi.org/10.1186/s40168-016-0181-2
Pascale, N., Gu, F., Larsen, N., Jespersen, L., & Respondek, F. (2022). The potential of pectins to modulate the human gut microbiota evaluated by in vitro fermentation: A systematic review. *Nutrients, 14*(17). https://doi.org/10.3390/nu14173629
Pezzali, J. G., Shoveller, A. K., & Ellis, J. (2021). Examining the effects of diet composition, soluble fiber, and species on total fecal excretion of bile acids: A meta-analysis. *Frontiers in Veterinary Science, 8*, Article 748803. https://doi.org/10.3389/fvets.2021.748803
Popov, S. V., & Ovodov, Y. S. (2013). Polypotency of the immunomodulatory effect of pectins. *Biochemistry. Biokhimiia, 78*(7), 823–835. https://doi.org/10.1134/S0006297913070134
Prado, S. B. R., Beukema, M.[. M.]., Jermendi, E., Schols, H. A.[. H. A.]., Vos, P.d.[. P.]., & Fabi, J. P. (2020). Pectin interaction with immune receptors is modulated by ripening process in papayas. *Scientific Reports, 10*(1), 1690. https://doi.org/10.1038/s41598-020-58311-0
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), Article D590-6. https://doi.org/10.1093/nar/gks1219
R Core Team. (2014). R: A language and environment for statistical computing. *MSOR Connections, 1*.
Rios-Covian, D., González, S., Nogacka, A. M., Arboleya, S., Salazar, N., Gueimonde, M., & de Los Reyes-Gavilán, C. G. (2020). An overview on fecal branched short-chain fatty acids along human life and as related with body mass index: Associated dietary and anthropometric factors. *Frontiers in Microbiology, 11*, 973. https://doi.org/10.3389/fmicb.2020.00973
Sahasrabudhe, N. M., Beukema, M.[. M.]., Tian, L., Troost, B., Scholte, J., Bruininx, E., … Vos, P.d.[. P.]. (2018). Dietary fiber pectin directly blocks toll-like receptor 2-1 and prevents doxorubicin-induced ileitis. *Frontiers in Immunology, 9*, 383. https://doi.org/10.3389/fimmu.2018.00383
Sarafian, M. H., Lewis, M. R., Pechlivanis, A., Ralphs, S., McPhail, M. J. W., Patel, V. C., … Nicholson, J. K. (2015). Bile acid profiling and quantification in biofluids using ultra-performance liquid chromatography tandem mass spectrometry. *Analytical Chemistry, 87*(19), 9662–9670. https://doi.org/10.1021/acs.analchem.5b01556
Schwartz, S. E., Levine, R. A., Singh, A., Scheidecker, J. R., & Track, N. S. (1982). Sustained pectin ingestion delays gastric emptying. *Gastroenterology, 83*(4), 812–817.
Shamji, M. H., Valenta, R., Jardetzky, T., Verhasselt, V., Durham, S. R., Würtzen, P. A., & van Neerven, R. J. J. (2021). The role of allergen-specific IgE, IgG and IgA in allergic disease. *Allergy, 76*(12), 3627–3641. https://doi.org/10.1111/all.14908
Sila, D. N., van Buggenhout, S., Duvetter, T., Fraeye, I., de Roeck, A., van Loey, A., & Hendrickx, M. (2009). Pectins in processed fruits and vegetables: Part II-structure-function relationships. *Comprehensive Reviews in Food Science and Food Safety, 8*(2), 86–104. https://doi.org/10.1111/j.1541-4337.2009.00071.x
Simpson, E. H. (1949). Measurement of diversity. *Nature, 163*(4148), 688. https://doi.org/10.1038/163688a0
Sun, Y., He, Y., Wang, F., Zhang, H.[. H.]., Vos, P.d.[. P.]., & Sun, J. (2017). Low-methoxyl lemon pectin attenuates inflammatory responses and improves intestinal barrier integrity in caerulein-induced experimental acute pancreatitis. *Molecular Nutrition & Food Research, 61*(4). https://doi.org/10.1002/mnfr.201600885
Tan, H., & Nie, S. (2020). Deciphering diet-gut microbiota-host interplay: Investigations of pectin. *Trends in Food Science & Technology, 106*, 171–181. https://doi.org/10.1016/j.tifs.2020.10.010
Tang, X., Beukema, M.[. M.]., Ferrari, M., Walvoort, M. T. C., de Haan, B. J., & Vos, P.d.[. P.]. (2023). Efficacy of pectins with different degrees of methyl-esterification and of blockiness in preventing gut epithelial cell barrier disruption and the impact on sodium-glucose co-transporter expression under low and high glucose conditions. *Food & Function, 14*(13), 6226–6235. https://doi.org/10.1039/d3fo01436c
Tian, L., Bruggeman, G., van den Berg, M., Borewicz, K., Scheurink, A. J. W., Bruininx, E., … Gruppen, H. (2017). Effects of pectin on fermentation characteristics, carbohydrate utilization, and microbial community composition in the gastrointestinal tract of weaning pigs. *Molecular Nutrition & Food Research, 61*(1). https://doi.org/10.1002/mnfr.201600186
Tian, L., Scholte, J., Borewicz, K., van den Bogert, B., Smidt, H., Scheurink, A. J. W., … Schols, H. A.[. H. A.]. (2016). Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats. *Molecular Nutrition & Food Research, 60*(10), 2256–2266. https://doi.org/10.1002/mnfr.201600149
Tian, Y., Gui, W., Koo, I., Smith, P. B., Allman, E. L., Nichols, R. G., … Patterson, A. D. (2020). The microbiome modulating activity of bile acids. *Gut Microbes, 11*(4), 979–996. https://doi.org/10.1080/19490976.2020.1732268
Tingirikari, J. M. R. (2018). Microbiota-accessible pectic poly- and oligosaccharides in gut health. *Food & Function, 9*(10), 5059–5073. https://doi.org/10.1039/c8fo01296b

Wai, W. W., Alkarkhi, A. F. M., & Easa, A. M. (2009). Optimization of pectin extraction from durian rind (*Durio zibethinus*) using response surface methodology. *Journal of Food Science, 74*(8), C637–C641. https://doi.org/10.1111/j.1750-3841.2009.01331.x

Weigmann, B., Tubbe, I., Seidel, D., Nicolaev, A., Becker, C., & Neurath, M. F. (2007). Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nature Protocols, 2*(10), 2307–2311. https://doi.org/10.1038/nprot.2007.315

Wickham, H. (2016). *ggplot2*. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-24277-4

Wilmore, J. R., Gaudette, B. T., Gomez Atria, D., Hashemi, T., Jones, D. D., Gardner, C. A., … Allman, D. (2018). Commensal microbes induce serum IgA responses that protect against polymicrobial sepsis. *Cell Host & Microbe, 23*(3), 302–311.e3. https://doi.org/10.1016/j.chom.2018.01.005

Xiao, F., Gao, X., Hu, H., Le, J., Chen, Y., Shu, X., … Zhang, T. (2022). Exclusive enteral nutrition exerts anti-inflammatory effects through modulating microbiota, bile acid metabolism, and immune activities. *Nutrients, 14*(21). https://doi.org/10.3390/nu14214463

Zandleven, J., Beldman, G., Bosveld, M., Schols, H. A.[. H. A.]., & Voragen, A. (2006). Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase. *Carbohydrate Polymers, 65*(4), 495–503. https://doi.org/10.1016/j.carbpol.2006.02.015

Zeng, M. Y., Cisalpino, D., Varadarajan, S., Hellman, J., Warren, H. S., Cascalho, M., … Núñez, G. (2016). Gut microbiota-induced immunoglobulin G controls systemic infection by symbiotic bacteria and pathogens. *Immunity, 44*(3), 647–658. https://doi.org/10.1016/j.immuni.2016.02.006

Zhao, S., & Li, L. (2018). Dansylhydrazine isotope labeling LC-MS for comprehensive carboxylic acid submetabolome profiling. *Analytical Chemistry, 90*(22), 13514–13522. https://doi.org/10.1021/acs.analchem.8b03435