

Allergen Immunization Induces Major Changes in Microbiota Composition and Short-Chain Fatty Acid Production in Different Gut Segments in a Mouse Model of Lupine Food Allergy

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Abstract

Background: The incidence of food allergies in western countries has increased in recent decades. **Objectives:** To study the association between gut bacterial microbiota composition, short-chain fatty acids (SCFAs) and food allergy in a mouse model. **Methods:** After oral immunizations with the human food allergen lupine with the adjuvant cholera toxin (CT) (or buffer in controls), sensitization and anaphylactic responses were determined. Gastrointestinal content was collected from the distal ileum, cecum, colon, and fecal pellets, and the bacterial diversity and composition was determined by deep sequencing of the 16S rRNA gene. SCFAs in gastrointestinal content supernatants were determined by gas chromatography. **Results:** The microbiota signatures were profoundly affected by allergen immunization. Ten operational taxonomic units (OTUs) were significantly different between immunized and control animals for at least one of the intestinal segments; eight of these OTUs belonged to the Clostridia class. Although consistent across all four gut seg-

ments, the colon showed the highest number of OTUs significantly associated with allergic immunization. SCFA levels in the cecum were also altered by immunization. **Conclusions:** Allergen immunization with CT in the present food allergy model induced profound changes in the microbiome composition and SCFA production. The result suggests that the colon may be the most sensitive gut segment for investigating changes in the gut microbiome.

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Introduction

Food allergies are emerging as a major public health challenge, particularly in western countries, with a reported prevalence of about 5–10% [1]. The increased incidence of allergic diseases has been linked to changes in human lifestyle and environmental factors, including changes in external and internal microbiota. It is now well established that the (gut) microbiota plays a pivotal role in the development of the human and animal immune

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system. Both reduced diversity and specific composition of the gut microbiome have been reported to be associated with the development of asthma, allergic rhinitis, and eczema in humans and in animal models [2–7]. Although less studied, the microbiome is also suggested to play a role in the development of food allergy [8–16].

Several mechanisms for how commensal bacteria exert immune modulation in food allergy have been suggested. Food allergy develops following loss of oral tolerance, which allows allergic sensitization [17]. Since barrier integrity and function is important to maintain oral tolerance to proteins, the known role of the microbiome in maintaining the barrier homeostasis is probably important to reduce allergen uptake and/or allergen interaction with the epithelial and immune cells [6, 18]. Further, metabolites produced by the microbiota can affect immune cell function directly, like for instance the reported effects of short-chain fatty acids (SCFAs) protecting against oral sensitization in mice by numerous pathways in the gastrointestinal tract, including IgA production, CD103+ dendritic cells [19], and regulatory T cell function [20]. Both the bacterial strain composition, e.g., clostridium [21, 22], and the dietary composition (like high fiber content, vitamin A, and probiotic supplements) [19, 22, 23] may positively contribute to SCFA production, thus providing beneficial effects in relation to allergy development.

Several mouse models of food allergy have been established for research purposes, and the various parameters influencing the character and intensity of the allergy endpoints are reviewed in Bøgh et al. [24]. However, in spite of the use of inbred mice, standardized maintenance, handling, and exposure to the animals in the same groups, there is a considerable inter-individual variation in immune responses. In several food allergy models, including ours, some of the immunized animals even appear to be non- or low responders, i.e., responding with no or weak anaphylaxis after allergen challenge and/or weaker allergen-specific IgE and IgG1 production than the rest of the group [25–29]. Further, food allergy responses have been reported to vary between labs [30] and the same strain of mice from different mouse providers [31] (and unpublished observations). Microbiota variations may contribute to these inter-individual and inter-lab variations in food allergy responses in mice, yet there is limited knowledge on how the microbiota influence food allergy models.

The majority of the studies on the microbiome, also in food allergy models, have been performed on the fecal pellet, while it is well known that the microbiome abun-

dancy, diversity, and composition change throughout the intestinal tract [32]. Alongside, the immune cell composition also varies throughout the intestines, and these regional variations in microbiota and immune cells will obviously influence the interactions between the two [32]. It has been suggested that the SCFAs, which are mainly produced by bacteria in the colon, may be absorbed and transferred from the colon to the intestines via blood and lymph circulation, there contributing to immune regulation (sensitization vs. tolerance) [18]. Little is known, however, about how the differences in microbial composition and SCFA production between allergic and control animals are reflected throughout the gastrointestinal tract.

The aim of this work was to determine the association between the gut bacterial microbiota composition, the gut SCFA levels, and sensitization and anaphylaxis in a lupine food allergy model in mice. Additionally, we investigated the microbiota variations at four different segments of the gastrointestinal tract, to identify which gut segment can best reflect the differences in microbial composition between allergic and control animals. The present results contribute to increased knowledge on the relation between gut bacterial composition, SCFAs, and food allergy. Increased knowledge about “pro- or anti-allergic” microbiome signatures may contribute to better standardization of and possibly adjuvant-free food allergy models, as well as future microbial intervention approaches for allergy prevention.

Materials and Methods

Study Design

The animal experiment in this work was part of a previous study, employing a mouse model of food allergy with lupin as the model allergen and the use of cholera toxin (CT) to break oral tolerance (Andreassen et al. [25], 2016). In brief, mice ($n = 11$ – 13 in each treatment group) were repeatedly exposed via oral gavage to Hank's Balanced Salt Solution (HBSS) buffer (vehicle control) and lupin + CT, and subsequently intraperitoneally challenged with lupin, according to the exposure regime described below. Fecal pellets and the content of the distal ileum (small intestine), cecum, and colon were collected from the mice in order to investigate the impact of the microbiome on (lupin) food allergy.

Animals

Five-week-old female C3H/HeJ mice (Jackson Laboratories, USA) were randomly allocated to groups ($n = 11$ – 13 per group) and housed in cages (3–5 animals together) in IVC racks, on Nest-pack bedding (Datesand Ltd, Manchester, UK). Harlan Teklad 2018 rodent diet and tap water were given ad libitum, in an environment with 12/12-h light/dark cycle, room temperature (RT) of $21 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ humidity. The experiment was performed

in accordance with the laws and regulations for live animals in Norway and approved by the Norwegian Animal Research Authority under the Ministry of Agriculture (FOTS ID 4534).

Animal Experiments

As previously described in Andreassen et al. [25] (2016), on days 0, 1, 2, 7, 21, and 28, each animal was intragastrically gavaged with 250 μ L of either HBSS (control group) or HBSS with 5.7 mg (mg/kg bw/day) lupin protein extract and 10 μ g of CT (immunized group). A 100- μ L blood sample was collected from the vena saphena lateralis from each animal on days 0 and 34. Before the blood collection on day 34, each mouse was kept in an empty container until 1–3 fecal pellets could be collected. The pellets were for practical reasons frozen at -80°C for 2 days, before being thawed in 200 μ L of STAR buffer (Roche Molecular Systems, Inc., Branchburg, NJ, USA) for 1–2 h, suspended by use of disposable plastic pipettes with wide opening, and frozen at -80°C until analysis.

Assessment of Clinical Anaphylactic Reactions and Collection of Gut Content

On day 35, the animals were intraperitoneally challenged with 250 μ L of HBSS containing 5 mg of the allergen. The animals were monitored for 30 min directly after the challenge, and given a score describing their clinical anaphylactic reactions, as described by Li et al. [33] (0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, pili erecti, inactivity or decreased activity with an increased respiratory rate; 3 = wheezing, labored respiration, cyanosis around the mouth and tail; 4 = no activity after prodding or tremor or convulsion; and 5 = death). Rectal temperature was measured after 0, 15, and 30 min using a BAT-12 microprobe thermometer (probe RET-3) from Physitemp Instruments Inc. (Clifton, NJ, USA). After the 30-min monitoring period (or if the animal displayed symptoms according to score 4) the mice were immediately anesthetized with 3.5% Isoflurane gas (Isoba vet; Intervet/Schering-Plough Animal Health, Lysaker, Norway) administered in surgical O_2 in an inhalation chamber before exsanguination by heart puncture. Blood was collected and serum prepared and frozen at -20°C . The content of the 12 lowest cm of the small intestine (distal ileum) and of the whole cecum and colon were harvested by flushing with approximately 1, 0.5, and 0.5 mL of ice-cold PBS for the ileum, cecum, and colon, respectively, and kept on ice until centrifuged at 3,000 rpm for 10 min. The supernatants were transferred to individual tubes and frozen at -80°C until SCFA analyses. The pellets were resuspended in 200–400 μ L of STAR buffer and stored at -80°C until microbiome analyses.

Assessment of Allergic Sensitization

Lupin-specific IgE antibodies were not measured due to technical challenges in using the lupin extract as an allergen source in ELISA, but allergen-specific IgG1 and total IgE in serum were determined and were previously shown to be closely related to the anaphylactic response to the allergen [25].

Detection of Lupin-Specific Antibodies

Lupin-specific IgG1 antibodies in serum were assessed as described by Vinje et al. [34]. In short, 96-well microplates coated with lupin extract (5 μ g/mL) were incubated for 1 h at RT and then overnight at 4°C . After washing (Tris/Tween), 1 h of blocking (Tris/Tween with 1% bovine serum albumin), and subsequent washing, lupin-specific IgG1 standard serum in fourfold dilutions

for standard curve generation, negative control serum, and the sera collected on day 34 (diluted 1:100 in BSA/Tris/Tween) were applied and incubated for 2 h at RT. After washing, incubation with peroxidase-labeled rat anti-mouse IgG1 antibodies at RT for 2 h, and subsequent washing, the plates were incubated for 15 min with peroxidase substrate. A BioTek Elx808 Absorbance Microplate reader at 405 nm using Gen5™ Microplate Data Collection & Analysis Software (BioTek® Instruments, Inc., Winooski, VT, USA) were used for data/plate reading.

Detection of Total IgE Antibodies

Total IgE concentrations in serum were determined in the blood samples collected on day 34, using a mouse IgE ready-set-go ELISA kit from Affymetrix eBioscience (Vienna, Austria) according to the manufacturer's instructions.

Microbiome Analyses

DNA Extraction

Gut content samples from the distal ileum (immunized $n = 13$, control $n = 11$), cecum (immunized $n = 13$ control $n = 11$), colon (immunized $n = 13$, control $n = 11$) and feces (immunized $n = 8$, control $n = 9$) were thawed and transferred to 2-mL microtubes (Sarstedt, Germany) prefilled with 0.2 g of acid-washed beads ($\leq 106 \mu\text{m}$; Sigma-Aldrich, Germany) before bead beating in a MagNA Lyser instrument (Roche, Switzerland). Bead beating was performed twice at 6,500 rpm for 20 s with 1-min rest between runs where samples were kept cold. DNA was extracted using a paramagnetic bead-based mag midi kit (LGC Genomics, UK) in a KingFisher Flex system (Thermo Fisher Scientific, USA) following the manufacturer's recommendations. The purified DNA was quantified using a Qubit dsDNA HS kit (Thermo Fisher Scientific), following the manufacturer's instructions.

16S rRNA Library Preparation and Sequencing

For analysis of prokaryote composition in the intestinal content, sequencing of the 16S rRNA gene was performed. Amplicons were obtained using a two-step PCR with primer pairs PRK341F (5'-CCTAC GGGRB GCASC AG-3')/PRK806R (5'-GGACT ACYVG GGTAT CTAAT-3') [35] targeting the V3-V4 region of the 16S rRNA gene. In first-step PCR, each reaction mix comprised 1.25 U of HotfirePol DNA polymerase (Solis BioDyne, Estonia), 1 $\times$ buffer B2, 2.5 mM MgCl_2 , 200 μM dNTP mix, 0.2 μM of each primer, and 1 μL of template DNA (20–40 ng). Thermal conditions were set to initial denaturation at 95°C for 15 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s. A single final elongation step at 72°C for 7 min was included. Resulting amplicons were cleaned using AMPure XP beads (Beckman Coulter, USA) before second-step PCR with modified primers required for sequencing. The reaction mix and thermal conditions in the second PCR were the same as previously described, only with 10 cycles and a prolonged annealing step (1 min). Quantification was done using a Qubit dsDNA HS kit (Thermo Fisher Scientific), following the manufacturer's instructions, before samples were normalized and pooled to a sequencing library. A final clean-up step using AMPure XP beads was included before the library was quantified using primers targeting Illumina adapters and EvaGreen Supermix (Bio-Rad, USA) in a QX200 Droplet Digital PCR system (Bio-Rad) following the manufacturer's recommendations. Sequencing was performed on a MiSeq platform (Illumina, USA) as instructed by the manufacturer.

Data Analysis

Resulting reads from the sequencing were analyzed using the QIIME pipeline [36] implementing UPARSE 8.0 operational taxonomic unit (OTU) clustering [37]. In short, the sequences were quality filtered (no mismatch in barcode allowed, Q score >25, singletons removed), demultiplexed, clustered if showing more than 97% sequence similarity, and assigned taxonomy using the Greengenes database 13_8 [38]. To obtain a uniform sequencing depth, 8,000 sequences from each sample were used for analysis.

SCFA Analyses

Detection and quantification of SCFA (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) in the supernatants from the distal ileum (immunized $n = 11$, control $n = 10$), cecum (immunized $n = 12$, control $n = 9$) and colon (immunized $n = 11$, control $n = 10$) were done using a Trace 1310 gas chromatograph (Thermo Fisher Scientific) equipped with an auto sampler, a flame ionization detector (FID), a split/splitless injector, and a Stabilwax DA column (30 m, 0.25 mm ID, 0.25 μm) (Restek, USA). Standard solutions were prepared by mixing formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and 2-methylvaleric acid in final concentrations, respectively, of 1%, 1,500 μM , 60 μM , 40 μM , 60 μM , 40 μM , 10 μM , and 125 μM , in Milli-Q water. All acids were purchased from Sigma-Aldrich, Germany.

To prepare samples for gas chromatography (GC) analysis, 200 μL of supernatants from flushing of intestinal segments were mixed with equal volumes of a solution containing 2% formic acid and 500 μM of internal standard (2-methylvaleric acid). The internal standard was included to control equal sampling volume introduced into the system by the syringe. Samples were then centrifuged at 13,000 rpm for 10 min before the supernatants were transferred to spin columns (0.2- μm filter; VWR, USA) and centrifuged at 10,000 rpm for 5 min. The eluate was then transferred to 300- μL GC vials (VWR) and analyzed in the GC-FID instrument. Samples were injected into the GC-FID system in split mode (2.5:200) in volumes of 0.2 μL . Injector and detector temperatures were set to 250 and 275 $^{\circ}\text{C}$, respectively, and helium was used as a carrier gas. Temperature intervals comprised a 2-min step at 90 $^{\circ}\text{C}$ followed by a 6-min increase to 150 $^{\circ}\text{C}$ and a 2-min increase to 245 $^{\circ}\text{C}$ followed by a hold at this temperature for 4.9 min. The software Chromeleon 7.2 (Thermo Fisher Scientific) was used for instrument control, quantification, and data analysis.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5. The nonparametric Mann-Whitney test was used to test differences between immunized and control mice. Differences between the three groups (control, low responders, and allergic mice) were tested with the nonparametric Kruskal-Wallis test, using Dunn's multiple comparison post hoc test to evaluate differences between the individual groups. p values <0.05 were considered statistically significant.

To compare the OTU levels between the two groups, the mean counts for each OTU (for each gut sampling site) in the immunized group minus the control group were divided by the sum of the mean values. The OTUs with a false discovery rate Q score <0.05 were regarded as statistically significantly different between the two treatment groups.

Results

Allergy-Related Endpoints after Allergen Immunization and Challenge

The allergen-immunized group had significantly higher lupin-specific IgG1 ($p < 0.0001$) and total IgE ($p = 0.0117$) levels (measured before allergen challenge) compared to the control group (Fig. 1a, b). Induction of clinical food allergy in the immunized group of mice was demonstrated as anaphylaxis after allergen challenge, measured as a group median anaphylactic score of 4 (75 percentiles 3 and 4) and a median temperature fall of 3 $^{\circ}\text{C}$ (75 percentiles -1.8 and -4 $^{\circ}\text{C}$) (Fig. 1c, d). The control group was, due to practical considerations, not challenged, but previous studies illustrate the lack of anaphylaxis after allergen challenge in nonimmunized mice [34, 39]. These allergy-related endpoints have previously been presented as a part of another study [25].

Microbiota Diversity and Composition Signatures after Allergic Immunization

The microbiome composition was determined in control and immunized animals, in material collected from the fecal pellet, the colon, the cecum, and the distal ileum. As shown in Figure 2, the strain diversity (alpha-diversity; Fig. 2a) and the overall bacterial class composition (Fig. 2b) were not profoundly affected by the allergen immunization when including samples from all gut segments. As established by previous studies, the bacterial microbiome in the ileum stood out from that of the three other segments both by demonstrating a lower diversity (Fig. 2c) and a different bacterial class composition (Fig. 2d). The main differences were an over-representation of the aerobic lactobacilli in the ileum.

When investigating the composition below the bacterial class level, the multivariate cluster analyses based on bacterial OTU levels in all the samples illustrate that both allergen immunization and the gut segment strongly influenced the gut microbiota composition (beta-diversity) (Fig. 3). Importantly, the principal component analysis (PCA) plot demonstrated a relatively clear clustering of the samples dependent on the bacterial OTU composition in the two groups of mice, i.e., the immunized and control animals (Fig. 3a; PC1 explaining 24.4% and PC2 explaining 12.6% of the total variation). The microbiome in immunized and control mice was not separated along PC1 (Fig. 3b) but statistically significantly so along PC2; Fig. 3c). This separation due to immunization was apparent in all four gut segments, illustrated by two clusters along PC2 in all segments (Fig. 3d, f). The PCA analyses

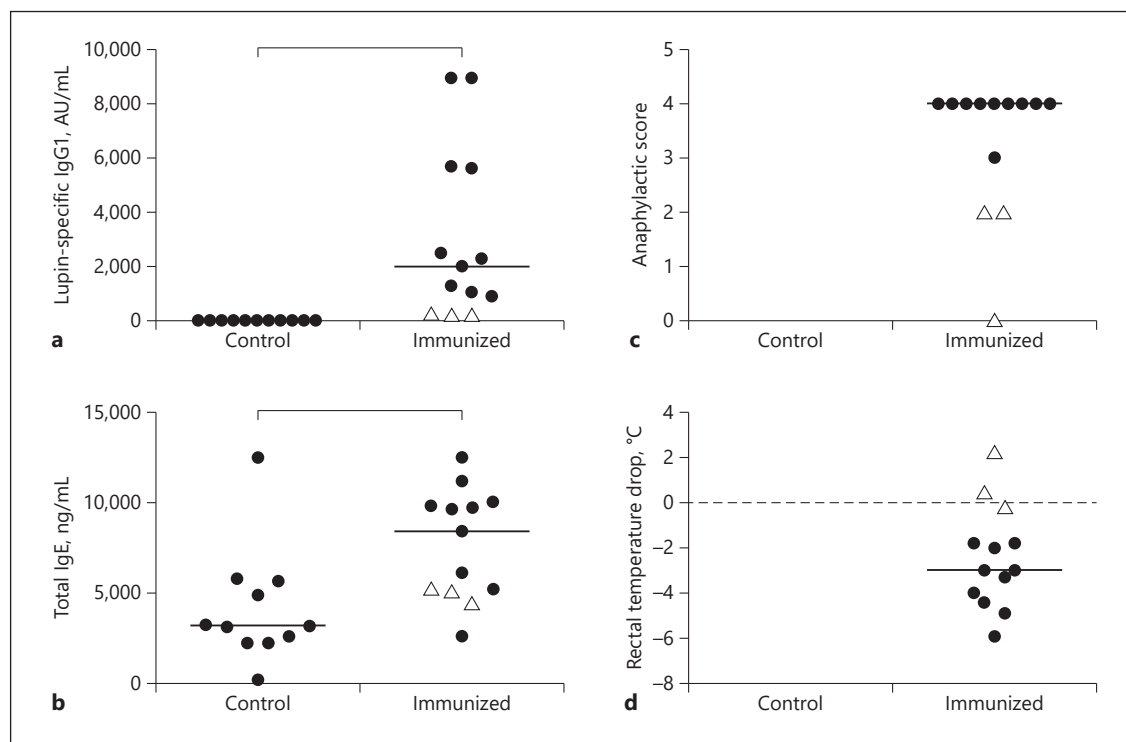


Fig. 1. Sensitization responses. Allergic sensitization expressed as lupin-specific IgG1 (**a**) and total IgE (**b**) in serum of immunized ($n = 13$) and control ($n = 11$) mice, and the anaphylactic score (**c**) and the drop in rectal temperature 30 min after allergen challenge (**d**) for the immunized mice. These data have previously been pre-

sented more thoroughly in Andreassen et al. [25] (2016). Points represent the individual animals (open triangles representing low-responding animals). The lines represent the group median, while the brackets denote statistically significant differences between groups ($p < 0.05$).

further illustrated how the OTU composition signatures differed between the ileum and the rest of the gut segments; observed as a clear clustering of the samples from the ileum, while no specific clustering was observed for the three other sampling positions (Fig. 3d, e). The gut segments explained the total OTU variation along the main component PC1 (Fig. 3e), while there was no difference between the gut segments for PC2 (Fig. 3f).

Particular Bacterial OTUs Associated with Allergic Immunization

Immunized versus control mice could be predicted based on the bacterial microbiota within each gut segment, since all gut segments showed high predictability of immunization based on the microbiota using PLS-DA analyses (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000492006). Thus, as a next step, we identified the OTUs significantly associated with the immunized versus the control group. We identified ten OTUs that were statistically significantly

different after allergen immunization within at least one of the gut segments (Fig. 4; group mean levels (%) for the ten OTUs are reported in online suppl. Table S1). For all the ten OTUs the directions of the associations with allergen immunization were consistent across all four gut segments, with three OTUs being negatively associated and seven positively associated with immunization. The most abundant OTUs affected were OTU 11 Lachnospiraceae (decreased by immunization from, on average, 8.7 to 2.4% for all segments), OTU 15 Lachnospiraceae, and OTU 19 Anaeroplasmata (increased from 0.7 to 4.6% and from 0 to 3.4% by immunization, respectively). All ten OTUs were significantly different in the two groups for the colon segment; thus, the samples from the colon had the highest number of OTUs significantly associated with allergen immunization. Further, the difference in OTU 75 belonging to the F16 family (CW040 class) was significant for all the gut segments except the fecal pellet, while OTU 19 belonging to the Anaeroplasmata family (Mollicutes class) was significant for all the segments ex-

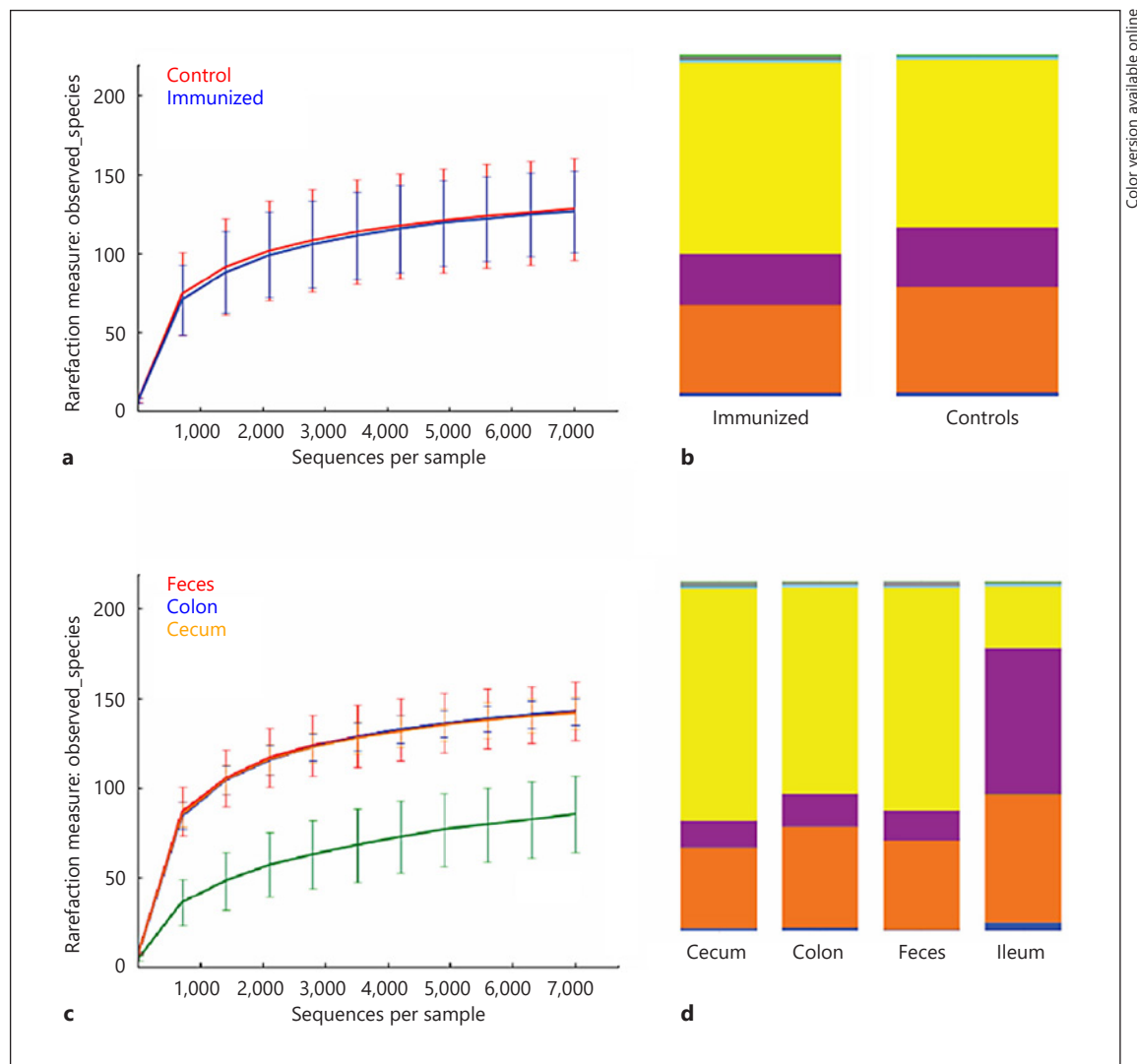


Fig. 2. Gut microbiota diversity and composition. Rarefaction curves (average $\pm$ SD) showing the species diversity (alpha-diversity) in immunized (blue) and control (red) animals (**a**), as well as in the four gut segments: distal ileum (green), cecum (orange), colon (blue), and fecal pellets (red) (**c**). The average bacterial class composition is shown for the two groups of mice (**b**) and in the

samples from the four gut segments (**d**). Data from all four gut segments from both immunized ($n = 13$) and control ($n = 11$) animals are included for all graphs. The three most abundant classes were Firmicutes Clostridia (yellow), Bacteroidetes Bacteroidia (orange), and Firmicutes Bacilli (purple).

cept the ileum (Fig. 4). The remaining eight OTUs belonged to the Clostridia class (Firmicutes phyla), with OTUs both positively and negatively associated with immunization.

Particular SCFAs Associated with Allergic Immunization and OTUs

The amount of the various SCFAs in the supernatants from the ileum samples was low (i.e., most samples were

below the detection limit; data not shown). In the cecum and colon segments, the abundance of the individual SCFAs measured were within a similar range (Fig. 5), while the composition (relative amount) differed between the two segments, like for instance the ratio of acetic to butyric acid. In the cecum, both acetic, isobutyric, and isovalenic acids were significantly higher in the immunized than in the control animals. No group differences were observed between any of the SCFAs in the colon.

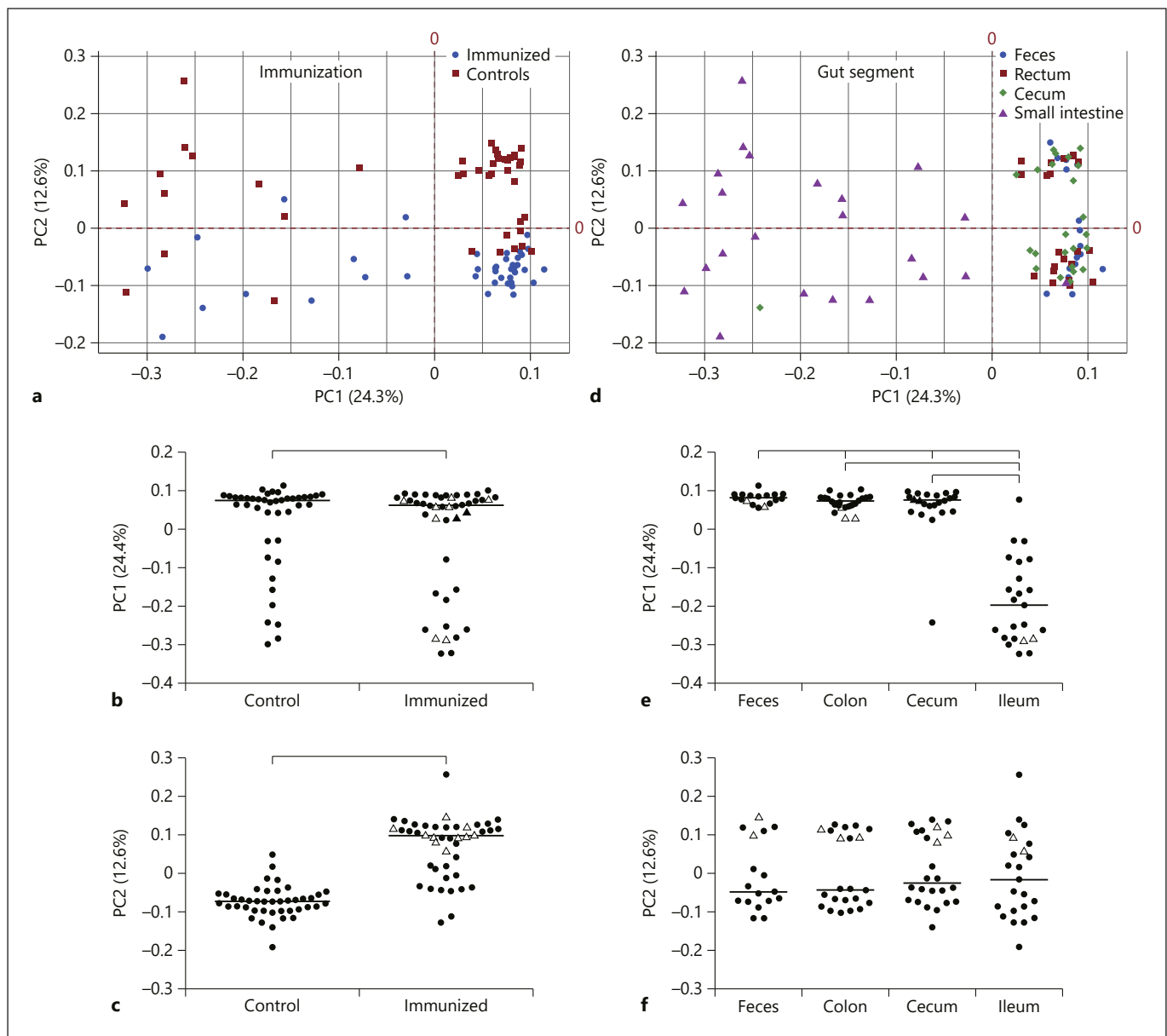


Fig. 3. The microbiome signature in relation to allergen immunization and gut segments. The upper panel (**a**, **d**) shows the weighted UniFrac plot showing the position of all samples from all animals (in total 96 samples) in relation to the two first principal components (PC), based on a PCA performed on the microbiome data for samples from the four different gut segments and from both immunized ($n = 13$) and control ($n = 11$) mice. Each dot represents one sample from one animal, color coded according to the legend

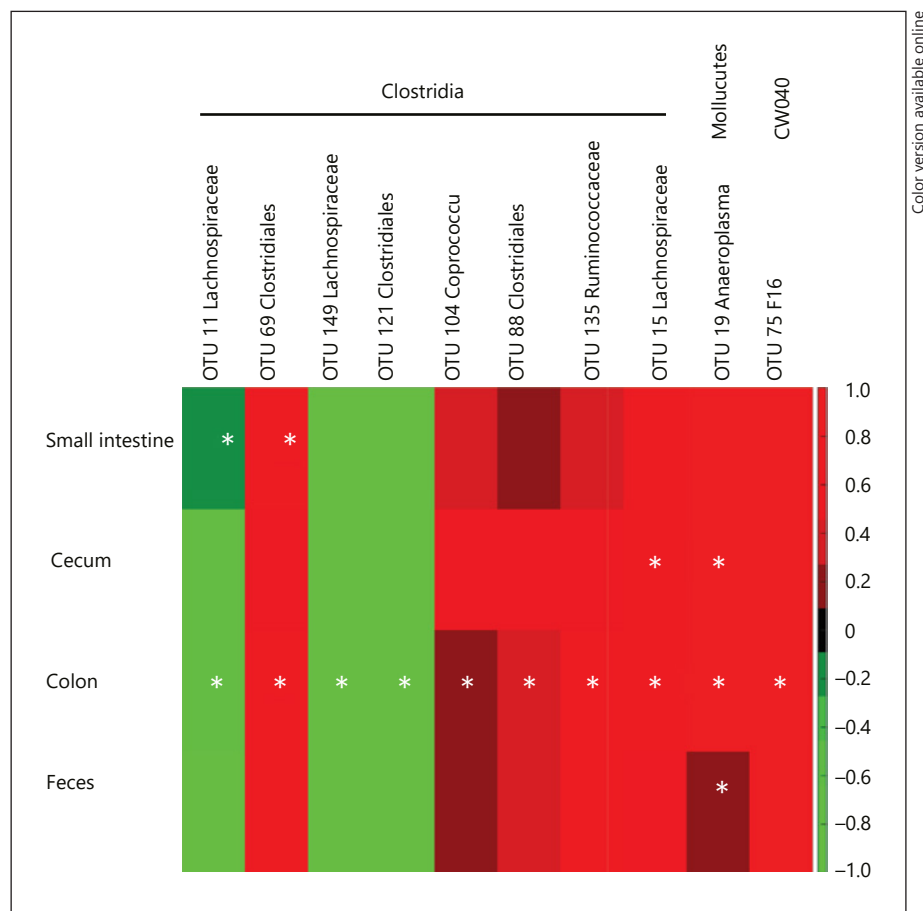
based on the treatment group (**a**) or gut segment sampling site (**d**). The lower panels further show how the values are positioned along PC1 and PC2 dependent on immunization (**b**, **c**) or gut segments (**e**, **f**). Points represent the individual animals (open triangles representing low-responding animals). The lines represent the group median, while the brackets denote statistically significant differences between groups ($p < 0.05$).

We were not able to establish an association between the ten immunization-associated bacteria OTUs and the SCFA levels in the respective gut segment (PLS regression analyses; results not shown).

Microbiota Composition in Immunized but Low-Responding Animals

Since previous studies have suggested that the gut bacterial composition may explain the protection from

Fig. 4. Associations between individual bacterial OTUs and immunization. The heatmap includes the OTUs (horizontally) that were significantly associated with immunization for at least one of the gut segments (distal ileum, cecum, colon, and feces; vertically). For each OTU, the mean counts for the immunized group minus the control group were divided by the sum of the mean values, and the result presented accordingly to the color scale on the right. Asterisks represent a FDR Q score <0.05. Positive (red color) values denote positive associations with the immunized group, while negative (green color) values denote inverse associations with the immunized group. The order of appearance of the OTUs is due to the occurrence in the OTU table. The bacteria class of the OTUs is indicated at the top of the figure.



food allergy in nonresponding individuals in various mouse allergy models [26], we performed preliminary analyses of this in the present study, even though the study was not designed with this aim. Three of the 13 animals that were orally immunized with lupin allergen and CT did demonstrate a statistically significantly weaker anaphylactic score than the rest of the immunized animals (scores 0–2 in low responders vs. scores 3–4 for allergic animals; $p = 0.0027$). They also did not show any temperature loss after lupin challenge (the changes in rectal temperature varied between -0.2 and $+2.3^{\circ}\text{C}$) as opposed to the responding animals ($p < 0.05$, temperature changes varied between -1.8 and -5.9°C). These low-responding animals also showed intermediate serum levels of lupin-specific IgG1 and total IgE antibodies (Fig. 1, triangles), although not statistically significantly different from the allergic responders or from control animals (probably due to the low number of animals). Thus, in spite of the same treatment as the other mice in the immunized group, these animals were low

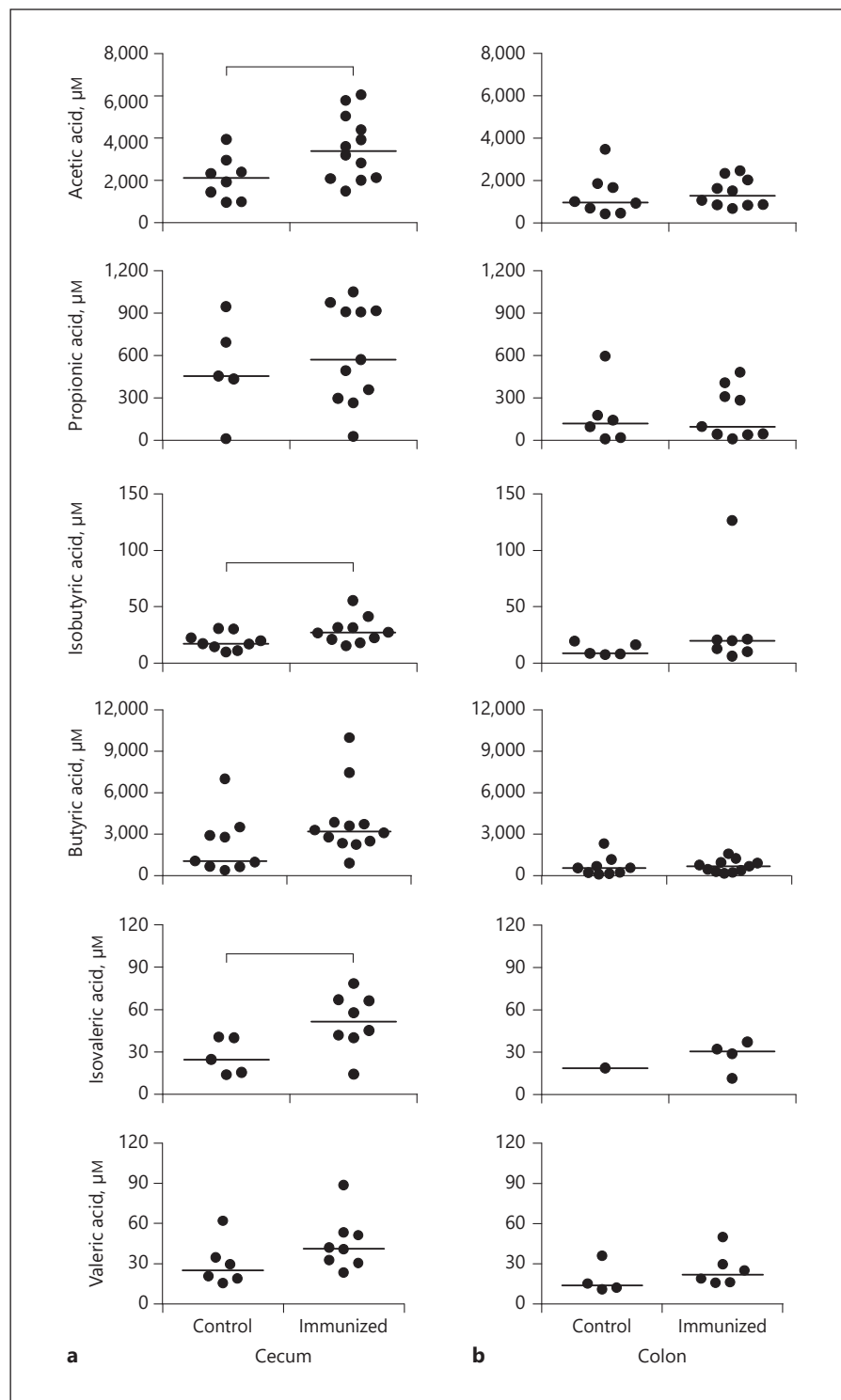
responders with regard to sensitization and anaphylaxis.

Looking at the bacterial composition signatures (Fig. 3c), immunized and control animals showed a clear separation along PC2, while within the immunized group there were no apparent difference between the low responders (triangles) and the allergic responders. This suggests that the overall bacterial signature was similar after immunization, irrespective of the animals being low responders or allergic after immunization.

Discussion

We report that immunization with allergen and CT in the present mouse model of lupin food allergy not only contributed to a profound change in bacteria composition signatures throughout the intestinal tract, but also changed the local SCFA composition. Most (eight of ten) of the significantly different bacteria strains belonged to

Fig. 5. SCFAs in colon and cecum supernatants. Concentrations of detectable SCFAs in the cecum (**a**) (immunized $n = 12$, control $n = 9$) and colon (**b**) (immunized $n = 11$, control $n = 10$) of mice are shown. Points represent the individual animals. The lines represent the group median, while brackets denote statistically significant differences between groups ($p < 0.05$).



the Clostridia class, while one belonged to the Mollicutes and one to the CW040 class. The changes in bacterial composition due to the immunization was most clearly expressed in the colon, but consistent throughout all seg-

ments, while the cecum showed the clearest differences in SCFA amounts and composition.

In our study, most of the OTUs that were significantly affected by immunization belong to the class of Clostrid-

ia (Fig. 2b, 4), in agreement with both human and animal studies, supporting an important role of this bacteria class in relation to allergy [15, 21, 22]. Clostridia bacteria are anaerobic spore-forming Firmicutes bacteria, which is a dominant class of commensal bacteria, residing in close proximity to the colonic epithelium [18]. This may explain why the colon was the most sensitive segment in which to detect changes for the Clostridia OTUs (see below). Germ-free mice colonized with a certain mix of human Clostridia have previously been shown to attenuate colitis and allergic diarrhea, and to be protected from allergic sensitization probably through induction of regulatory T cells in the colon and induction of IL-22 production, promoting gut barrier function [18, 21, 40]. Importantly, in our study, OTUs from the Clostridia class were both positively and negatively associated with allergic immunization (Fig. 4). The most abundant of the affected OTUs were from the Lachnospiraceae family (OTU 11 decreasing and OTU 15 increasing), in accordance with previous report of reduced Lachnospiraceae bacteria in a different animal model of food allergy [15]. Together with previous reports of varying potential for the induction of Tregs or Th17 cells in the colon by different Clostridia classes [21], this illustrates the need for more detailed knowledge of the bacterial families before the possible utilization of Clostridia in health-promoting interventions.

In addition to alterations in bacterial composition, also the overall levels of the SCFAs acetic, isobutyric, and isovaleric acids in the cecum were significantly different in immunized and control animals (Fig. 5). These changes in SCFA may be related to the profound alteration in the Clostridia species observed since Lachnospiraceae, and especially Clostridium cluster XIVa, are in general known as major producers of SCFA [19, 41]. On the other hand, it has become clear that not all members of this family have this SCFA-producing capacity [42]. Although we found associations between immunization and bacteria OTUs and immunization and several SCFAs, we were not able to establish a link between immunization, the affected bacterial OTUs, and SCFA production.

The increased levels of SCFAs in the immunized group were unexpected in the context of the previously suggested preventive effect of SCFA on food allergy development. This may, however, be explained by different experimental designs, since the present SCFA levels were measured in animals after immunization, while the protective effects were reported in animals with altered microbiome composition and/or SCFA status before immunization. Mouse strain, microbial composition of the

animal facilities, sampling site, and time also influence the results. Our observation may suggest that mechanisms other than SCFA were dominating the interactions between the microbiota and the immune system in our model. It should be noted, however, that the measurements only reflect the amounts of free SCFA in the intestinal content, and the observed higher amounts could reflect either increased production or reduced uptake by tissue in the different intestinal segments. Thus, the precise SCFA levels in the mucosal tissue or in serum, available for immune cell contact, are unknown and are difficult to quantify in vivo.

As expected from current knowledge, the microbiota in the distal ileum differed from the other segments with regard to diversity and composition (Fig. 2c). Although not statistically significant between immunized and control animals for all positions, we observed a consistent pattern for the ten affected bacterial strains (OTUs) throughout the ileum, cecum, colon, and fecal pellet samples, suggesting that the commonly used fecal pellets will reflect relevant changes in the intestinal microbiome. Immunization induced the most pronounced changes for OTUs in the colon, and for SCFA in the cecum (Fig. 4). The sensitization to food allergens, on the other hand, is thought to occur in the small intestines, where important immune players like CD103+CD11b+ dendritic cells, and ILCs, IELs, eosinophils, and mast cells are most prominent, while other cell types are more abundant in the colon (Tregs, IgA+ plasma cells, and macrophages [18, 32]. Previous reports support that the microbiota and SCFA composition in the colon is important for the Treg-promoting mechanisms of the gut microbiome [18]. How these changes in the colon affect sensitization and/or tolerance induction in the small intestines is largely unknown, but Cao et al. [18] hypothesized that cells, cytokines, and SCFA may be transferred from the colon to the intestines via blood and lymph circulation, contributing to reduced sensitization. Our observation that the changes in microbiota composition were consistent in the colon and the distal ileum (small intestines) may also support their hypothesis that the presence of Clostridia or other bacteria may also directly interact with the immune cells in the small intestines.

Non- or low-responding animals are often reported in food allergy mouse models, as demonstrated for 3 of the 13 immunized animals in the present study. The tolerance mechanisms are unknown, but a previous study has suggested that a different gut bacterial composition was associated with protection from food allergy in the low-responding individuals [26]. For exploration, even though

the number of nonresponders was low and this was not our initial aim, we investigated the microbiota composition of the low-responding animals in the immunized group. The overall bacterial signature was similar in immunized animals, irrespective of the animals being low responders or allergic after allergen and CT immunization. This indicates that the main bacterial microbiota signature was not associated with the diverging immunological responses in the low-responding animals. We cannot, however, exclude that inherent differences in the microbiome before immunization may have contributed to prevent sensitization and anaphylaxis in the low responders.

A clear limitation of the present study is that the effects of CT or the lupine allergen alone on the gut microbiome was not investigated, but rather the combined effect of the adjuvant and the allergen. Environmental exposure to food allergens is essential for the development of sensitization, but what has emerged clearly in the literature in recent years is that the adjuvants to which an individual is exposed at the same time as the allergen are probably an equally important determinant of the immune response to the allergen [43]. Since food allergy is a public health problem in humans, with a prevalence of between 5 and 8%, adjuvants able to break oral tolerance are probably quite abundant. Allergies have been associated with altered diversity and composition of gut microbiota [2–4, 8], and we hypothesize that effects by adjuvants on the microbiota composition are one of their mode of action. In the present model we used CT, a commonly used adjuvant in animal studies of food allergy [24], serving as a model adjuvant, since oral exposure to the allergen alone does not induce food allergy. Increased knowledge on the mechanisms on how adjuvants, including model allergens like CT, contribute to allergy development may open avenues for the identification of compounds (relevant for human exposure) with similar mechanisms of action. Few adjuvants relevant for humans have been identified in animal models, but include the mycotoxin deoxynivalenol (DON) [28]. CT has previously been reported to affect the bacterial composition in the gut, but with no profound effects on the Clostridia strains [44], the strains mostly affected in the present study. This suggests that the observed effects on Clostridia composition was caused by the allergen alone or in combination with CT.

Our data provide extended knowledge on how profound changes in the microbiota composition, predominantly the Clostridia class, are linked to food allergic sensitization and anaphylaxis after oral immunization. While the effects of immunization were consistent for samples

from the ileum, cecum, colon, and fecal pellet, our data suggest that colon material may be the most sensitive sampling point for bacterial investigations, unless time trends are to be performed and sampling pellets are the only option. The observed strong associations between food allergy and gut microbiota composition, as well as SCFA production, illustrates that the potential for interventions affecting the microbiome composition and/or its functional metabolites deserves further investigations in the setting of disease prevention.

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Statement of Ethics

The experiment was performed in accordance with the laws and regulations for live animals in Norway and approved by the Norwegian Animal Research Authority under the Ministry of Agriculture (FOTS ID 4534).

Disclosure Statement

The authors declare no conflict of interest.

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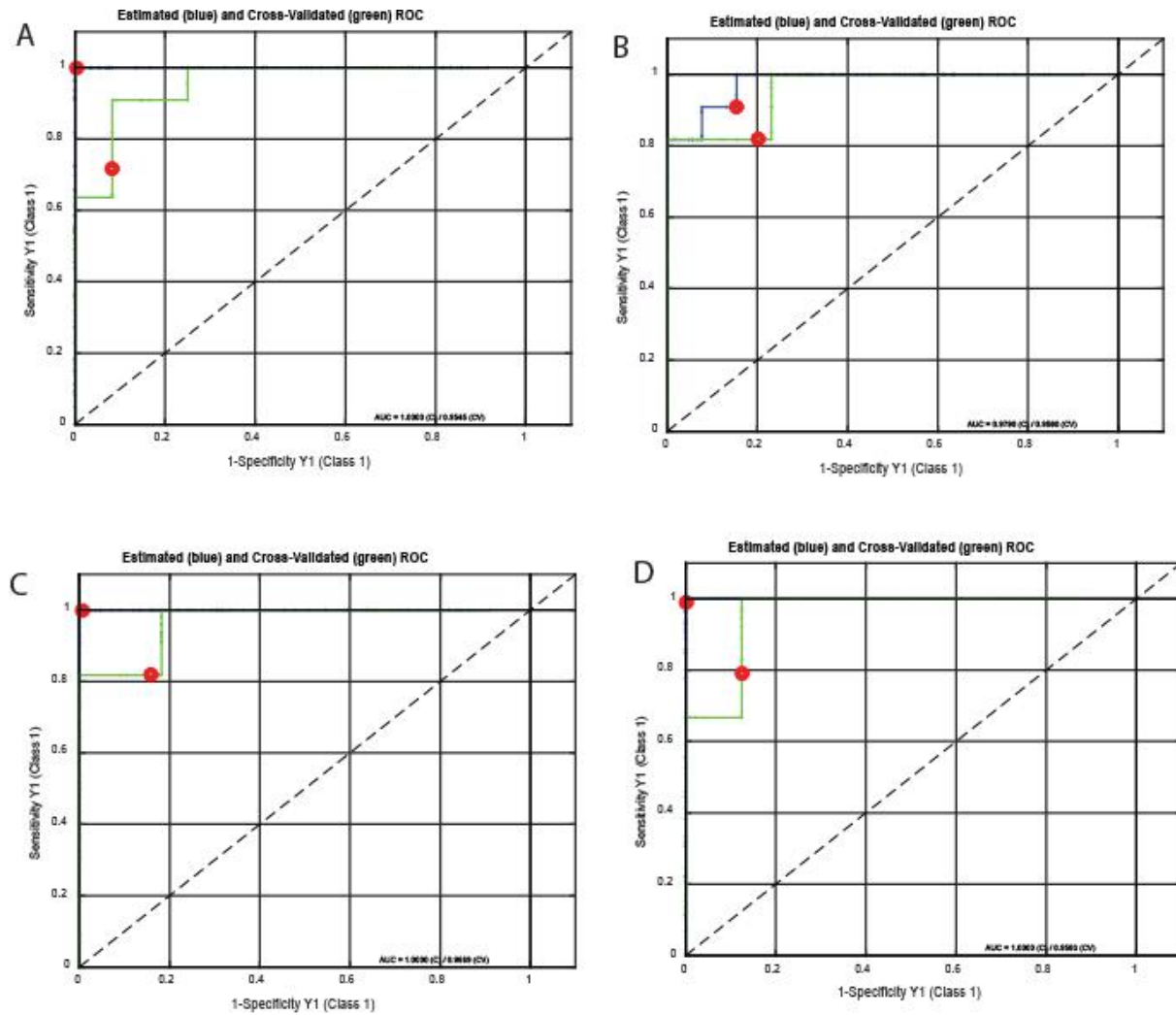
Author Contributions

M.A. and U.C.N. designed the study, performed the animal experiments, and wrote the manuscript. K.R. and I.L.A. performed the microbiome analysis. All authors contributed on the interpretation of results. All authors read and approved the final manuscript.

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Suppl Fig. S1. ROC curves for (A) small intestine. (B) cecum. (C) colon and (D) feces. Calibration values are shown as blue curves while validation values are shown with green curves.

Suppl. Table S1. Abundancy of the OTUs associated with immunization

OTU	Group	Ileum %	Cecum %	Colon %	Feces %	Sum %
OTU11 Lachnospiraceae	Control	0.33	2.99	3.10	2.33	8.74
	Immunised	0.02	0.54	0.45	1.43	2.43
OTU69 Clostridiales	Control	0.02	0.08	0.10	0.02	0.22
	Immunised	0.15	0.38	0.36	0.19	1.08
OTU149 Lachnospiraceae	Control	0.02	0.14	0.08	0.17	0.42
	Immunised	0.00	0.00	0.00	0.00	0.01
OTU121 Clostridiales	Control	0.10	0.17	0.50	0.27	1.04
	Immunised	0.00	0.00	0.00	0.00	0.00
OTU104 Coprococcu	Control	0.13	0.05	0.03	0.04	0.24
	Immunised	0.21	0.08	0.09	0.07	0.45
OTU88 Clostridiales	Control	0.07	0.08	0.04	0.04	0.23
	Immunised	0.16	0.17	0.16	0.05	0.55
OTU135 Ruminococcaceae	Control	0.00	0.01	0.03	0.04	0.08
	Immunised	0.01	0.05	0.11	0.08	0.24
OTU15 Lachnospiraceae	Control	0.04	0.28	0.24	0.24	0.79
	Immunised	0.14	1.64	1.57	1.26	4.60
OTU19 Anaeroplasma	Control	0.00	0.00	0.00	0.00	0.00
	Immunised	0.00	1.06	0.81	1.57	3.44
OTU75 F16	Control	0.00	0.00	0.00	0.00	0.00
	Immunised	0.30	0.09	0.16	0.19	0.74

Group mean level (in percentage) of the ten OTUs positively or negatively associated with the allergen immunization in the different gut segments (ileum, cecum, colon and feces) from control (n= 11) and immunized (n= 13) mice.