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Altered microbial community structure and metabolism in cow's milk allergic mice treated with oral immunotherapy and fructo-oligosaccharides

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RESEARCH ARTICLE

Abstract

Previously, we showed enhanced efficacy of oral immunotherapy (OIT) using fructo-oligosaccharides (FOS, prebiotics) added to the diet of cow's milk allergic mice indicated by a reduction in clinical symptoms and mast cell degranulation. Prebiotics are fermented by gut bacteria, affecting both bacterial composition and availability of metabolites (i.e. short-chain fatty acids (SCFA)). It is thus far unknown which microbial alterations are involved in successful outcomes of OIT with prebiotic supplementation for the treatment of food allergy. To explore potential changes in the microbiota composition and availability of SCFA induced by OIT+FOS. C3H/HeOuJ mice were sensitised and received OIT with or without a FOS supplemented diet. After three weeks, faecal samples were collected to analyse gut microbiota composition using 16S rRNA sequencing. SCFA concentrations were determined in cecum content. FOS supplementation in sensitised mice changed the overall microbial community structure in faecal samples compared to sensitised mice fed the control diet (P=0.03). In contrast, a high level of resemblance in bacterial community structure was observed between the non-sensitised control mice and the OIT+FOS treated mice. OIT mice showed an increased relative abundance of the dysbiosis-associated phylum Proteobacteria compared to the OIT+FOS mice. FOS supplementation increased the relative abundance of genus *Allobaculum* (*Firmicutes*), putative butyrate-producing bacteria. OIT+FOS reduced the abundances of the genera's unclassified *Rikenellaceae* (*Bacteroidetes*, putative pro-inflammatory bacteria) and unclassified *Clostridiales* (*Firmicutes*) compared to sensitised controls and increased the abundance of *Lactobacillus* (*Firmicutes*, putative beneficial bacteria) compared to FOS. OIT+FOS mice had increased butyric acid and propionic acid concentrations. OIT+FOS induced a microbial profile closely linked to non-allergic mice and increased concentrations of butyric acid and propionic acid. Future research should confirm whether there is a causal relationship between microbial modulation and the reduction in acute allergic symptoms induced by OIT+FOS.

Keywords: microbiota, food allergy, short-chain fatty acids, proteobacteria

1. Introduction

To date, food allergy management relies on strict allergen avoidance and symptomatic treatment to control adverse clinical events upon accidental exposure. A significant amount of antigen-specific immunotherapy trials to treat IgE-mediated food allergies shows promising results, especially using the oral route of administration, i.e. oral

immunotherapy (OIT) (Pajno *et al.*, 2017). A recent review discussing advances in OIT to treat peanut, hen's egg and cow's milk allergies concluded that overall achievement of clinical desensitisation was the most successful in OIT compared to sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT) and the highest tolerable dose of antigen could be consumed upon OIT (Burks *et al.*, 2018). Nevertheless, OIT causes the highest numbers of adverse events during and after treatment compared to SLIT and EPIT and thus needs to be improved (Burks *et al.*, 2018). The use of immunomodulatory (dietary) adjuvants with beneficial effects on gut health or immune regulatory processes might support skewing of the allergic immune response during OIT.

In previous work, we showed the added value of a diet supplemented with fructo-oligosaccharides (FOS, prebiotics) in supporting OIT efficacy to treat cow's milk allergy (CMA) in mice (Vonk *et al.*, 2017). OIT+FOS treatment improved desensitisation shown by a reduction in clinically related symptoms and mast cell degranulation (Vonk *et al.*, 2017). The beneficial effects of prebiotic supplementation with regard to either prevention or treatment of atopic disorders including eczema, allergic asthma and food allergy have been appreciated in both human and animals studies (McKenzie *et al.*, 2017; Schouten *et al.*, 2012; Sjodin *et al.*, 2016; Wopereis *et al.*, 2018; Yasuda *et al.*, 2010). In addition, it has been described in literature that prebiotic fibers protect against peanut allergy in mice via bacterial fermentation into short-chain fatty acids (SCFA) which enhance oral tolerance induction to peanut (Tan *et al.*, 2016). However, the contribution of prebiotic-mediated modulation of the microbial composition and metabolism in the gut during OIT to treat CMA remains to be elucidated. The enhanced efficacy observed upon OIT+FOS treatment in cow's milk allergic mice might be initiated by specific alterations at the microbiota level in the gut.

The human gut is colonised with bacterial species that belong to the following main phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and the *Fusobacteria* (Eckburg *et al.*, 2005). Bacterial communities feature complex metabolic activities and their interaction with the human host ensures both states of health and disease (Peterson *et al.*, 2015). *Firmicutes* and *Bacteroidetes* are the two major bacterial phyla that comprise the majority of the normal gut microbiota of healthy humans. Altered ratio of *Firmicutes* to *Bacteroidetes* has been linked to a variety of pathologies associated with aging or disease (Mariat *et al.*, 2009; Sokol *et al.*, 2009). In addition, the gut microbial composition of healthy humans is recognised by a low abundance of *Proteobacteria* (Shin *et al.*, 2015). Expansion of *Proteobacteria* members is linked to gut dysbiosis and a pro-inflammatory state in the host as shown for inflammatory bowel disease and Parkinson's disease (Keshavarzian *et al.*, 2015; Morgan *et al.*, 2012). Furthermore, airway microbial dysbiosis was shown to be related to exacerbations in severe asthmatics and was dominated by increased abundance of *Proteobacteria* in the lower airways (Huang *et al.*, 2015).

Considering (food) allergy development, the importance of the microbiome has been recognised in the concept of the 'hygiene hypothesis' (Strachan, 1989). *In utero* or post-natal exposure to environmental factors, such as maternal or infant diet and invading pathogens, shapes the gut microbiota composition and functionality with long-term effects on allergy or asthma development (McKenzie *et al.*, 2017; Stein *et al.*, 2016). Differences in microbial diversity and abundance of specific taxa were observed in infants showing early-life sensitisation against egg proteins or actual development of egg allergy (Fazlollahi *et al.*, 2018). Furthermore, resolution of CMA at 8 years of age was associated with enrichment of the phylum *Firmicutes* in infant gut microbiota samples collected at 3 to 6 months of age (Bunyavanich *et al.*, 2016). Manipulation of the bacterial composition has gained interest in the field of food allergy management: cow's milk allergic infants fed an extensively hydrolysed casein formula with the probiotic *Lactobacillus rhamnosus* GG showed accelerated tolerance induction after 6 months (Berni Canani *et al.*, 2016). Tolerant infants showed a significant increase in the abundance of *Oscillospira* (*Firmicutes*) compared to allergic infants and had elevated butyric acid levels in faecal samples (Berni Canani *et al.*, 2016).

The aim of the current experiment was to analyse the gastrointestinal tract's microbiota composition and metabolic activity-related SCFA profiles in cow's milk allergic mice subjected to the combination of OIT and FOS supplementation that had previously shown enhanced reduction of acute allergic symptoms upon food challenge (i.e. reduced acute allergic skin response, reduced symptoms of anaphylaxis and reduced mucosal mast cell degranulation) (Vonk *et al.*, 2017). The current data contribute to our understanding of the involvement of microbial alterations in the enhanced efficacy of OIT mediated by a prebiotic diet in the context of food allergy management.

2. Materials and methods

Animals

Female specific-pathogen-free C3H/HeOuJ mice (6-week old) were purchased (Charles River Laboratories, Erkrath, Germany) and randomly allocated to the following groups: sham-sensitised control, sham (n=5); whey-sensitised control, sens (n=8); FOS supplementation group, FOS (n=8); oral immunotherapy group, OIT (n=8); and the combination group of oral immunotherapy and FOS supplementation,

OIT+FOS (n=8). All mice were acclimatised for six days prior to the first sensitisation and were housed in filter-topped macrolon cages (one cage/group) at the animal facility of Utrecht University on a 12 h light/dark cycle with unlimited access to food and water. All experimental procedures were approved by the Ethical Committee of Animal research of Utrecht University (DEC.2014. III.12.120) and conducted according to the principles of good laboratory animal care as stated in the European Directive for the protection of animals used for scientific purposes.

Control and experimental diets

Upon arrival and during acclimatisation and oral sensitisation, all mice received the semi-purified cow's milk protein-free pelleted AIN-93G control diet. After oral sensitisation, mice in the FOS and OIT+FOS groups were fed the AIN-93G diet in which a specific mixture of plant-derived short-chain fructo-oligosaccharides (scFOS: oligofructose, Raftilose P95, degree of polymerisation (DP) <6) and long-chain fructo-oligosaccharides (lcFOS: long-chain inulin, Raftiline HP, average DP of 23 or higher with <1% DP of 5 or lower) was added in a 9:1 scFOS/lcFOS ratio (1% w/w) by Ssniff Spezialdiäten GmbH (Soest, Germany) as specified previously (Vonk *et al.*, 2017). Both scFOS and lcFOS were provided by Orafti (Wijchen, the Netherlands).

Sensitisation, oral immunotherapy and faecal sample collection

According to the experimental set-up depicted in Figure 1, all mice were orally sensitised (per gavage) against the cow's milk protein whey (DMV International, Veghel, the Netherlands) on day 0, 7, 14, 21 and 28 (20 mg whey in 0.5 ml phosphate buffered saline (PBS) with 15 µg cholera toxin (CT, List Biological Laboratories Inc., Campbell, CA, USA) per mouse). The sham-sensitised control group received CT in PBS alone. From D42-D59, mice in the OIT and OIT+FOS groups were subjected to oral dosing with 10 mg whey in 0.5 ml PBS (5×/week for 3 consecutive weeks) per gavage. After three weeks of dietary FOS supplementation and OIT, faecal samples were collected and the mice were sectioned in order to isolate cecum content at D63. In particular, to collect faecal samples, mice were individually housed for a short period of time and faecal pellets were collected using tweezers cleaned with ethanol. Faecal pellets in eppendorf tubes were immediately stored at dry ice for transportation. Subsequently, samples were stored at -80 °C until further processing. The findings in the current manuscript describing the faecal microbiota composition and bacterial metabolite analysis in caecum content are based on the samples collected in the previously published study (Vonk *et al.*, 2017).

Microbiota profiling and bioinformatics analysis

Total DNA was extracted from mice faeces collected after three weeks of experimental interventions utilising the FastDNA bead-beating Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), and verified with fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY). Primers 515FB/806RB (515FB:GTGYCAGCMGCCGCGGTAA; 806RB:GGACTACNVGGGTWTCTAAT) targeting the V4 variable region of microbial small subunit (SSU or 16S) ribosomal RNA (rRNA) genes were used for PCR (Walters *et al.*, 2016), and prepared for high-throughput amplicon sequencing using a modified two-step targeted amplicon sequencing (TAS) approach, as described previously (Green *et al.*, 2015). Negative controls were used with each set of amplifications, which indicated no contamination. Samples were pooled in equal volume using an EpMotion5075 liquid

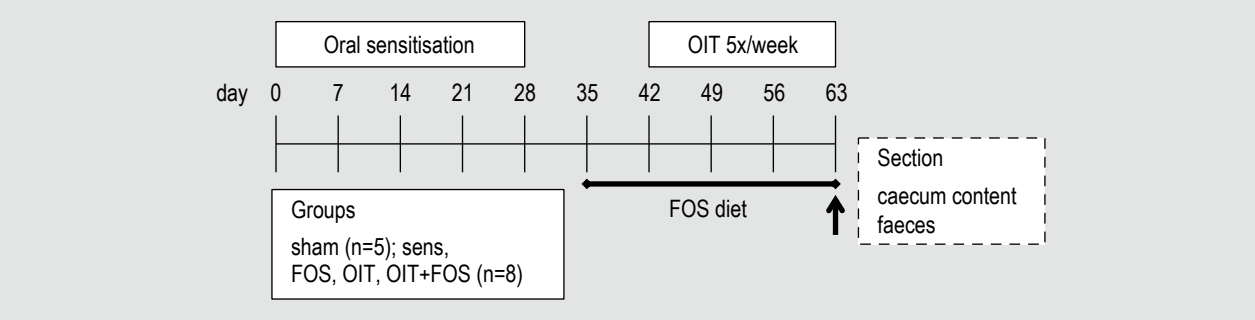

Figure 1. Experimental timeline. Female C3H/HeOuJ mice were fed the AIN-93G control diet and grouped as depicted: sham, sham-sensitised control (n=5); sens, whey-sensitised control (n=8); FOS, fructo-oligosaccharides supplementation group (n=8); OIT, oral immunotherapy group (n=8); OIT+FOS, combination of OIT and FOS supplementation group (n=8). On experimental day 0, 7, 14, 21 and 28, mice were sensitised per oral gavage to the cow's milk protein whey using cholera toxin (CT) as an adjuvant (20 mg whey in 0.5 ml PBS with 15 µg CT/mouse). Sham-sensitised mice received 0.5 ml PBS with 15 µg CT alone. On day 35, FOS and OIT+FOS mice were fed the AIN-93G diet supplemented with 1% FOS until the end of the protocol. OIT was provided per oral gavage from day 42-59 (10 mg whey in 0.5 ml PBS, 5×/week for 3 weeks). On day 63, faecal samples were collected and mice were sectioned to collect caecum content.

handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an Agencourt AMPure XP cleanup protocol (0.6×, v/v; Beckmann-Coulter, Brea, CA, USA) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell (2×153 paired-end reads; San Diego, CA, USA) and sequenced using Fluidigm sequencing primers. Based on the distribution of reads per barcode, the amplicons (before purification) were re-pooled to generate a more balanced distribution of reads. The re-pooled and re-purified libraries were then sequenced on a high-output MiniSeq run. Library preparation, pooling, and MiniSeq sequencing were performed at the DNA Services (DNAS) facility, Research Resources Center (RRC), University of Illinois at Chicago (UIC). Raw sequence data (FASTQ files) were deposited in the NCBI Sequence Read Archive under project PRJNA434262.

Raw FASTQ files for each sample were merged using the software package PEAR (Paired-end-read merger) (v0.9.8) (Schmieder and Edwards, 2011; Zhang *et al.*, 2014). Merged reads were quality trimmed and sequences shorter than 250 bases were discarded (CLC Genomics Workbench, v10.0; CLC Bio, Qiagen, Boston, MA, USA). Sequences were screened for chimeras (usearch8.1 algorithm) (Edgar, 2010), and putative chimeric sequences were removed from the dataset (QIIME v1.8) (Caporaso *et al.*, 2010). Each sample was rarefied (47,000 sequences/sample) and data were pooled, renamed, and clustered into operational taxonomic units (OTU) at 97% similarity (usearch8.1 algorithm). Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference database). A biological observation matrix (BIOM) (McDonald *et al.*, 2012) was generated at each taxonomic level from phylum to species ('make OTU table' algorithm) and analysed and visualised using the software packages Primer7 (Clarke, 1993) and the R programming environment (R Core Team, 2013).

Alpha-diversity (α-diversity) indices (within-sample) and beta-diversity (β-diversity) (between-sample) were used to examine changes in microbial community structure between mice faecal group samples. Alpha-diversity indices (i.e. Shannon, Simpson, richness, and evenness) were generated using the package 'vegan' implemented in the R programming language (Oksanen, 2016). To examine β-diversity differences in microbial community composition between samples, pairwise Bray-Curtis dissimilarity (non-phylogenetic) metric was generated using the Primer7 software package and used to perform analysis of similarity (ANOSIM) calculations. ANOSIM was performed at the taxonomic level of genus, using square-root transformed data. Also, Primer7 was used to conduct both non-metric multi-dimensional scaling (nMDS) and Bootstrapping (average values and dispersion within each sample's group) plots to visualise each mice group's overall microbial differences, at the genus level.

Beta-diversity differences in relative abundance of individual taxa, between mice faecal group samples, were assessed for significance using Kruskal-Wallis test controlling for false-discovery rate (FDR), implemented within the software package QIIME (Caporaso *et al.*, 2010). Furthermore, mice faecal group sample's community functional predictions were performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille *et al.*, 2013) and differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) abundances between groups were identified (Kanehisa and Goto, 2000).

Short chain fatty acid analysis in caecum content

SCFA analysis was conducted as described elsewhere (De Theije *et al.*, 2014). In short, caecum content was collected and stored at -80 °C until further processing. Samples were defrosted, diluted in ice cold PBS (1:10) and homogenised by vortexing. Supernatant was collected after centrifugation at 13,000 rpm for 10 min and analysed using the Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) and concentrations of acetic acid, butyric acid, propionic acid, valeric acid, iso-butyric acid and iso-valeric acid were quantitated based on 2-ethylbutyric acid internal standard.

Data analysis and statistics

Alpha- and β-diversity were measured at each taxonomic level from phylum to genus. In SPSS (v.22, IBM, Chicago, IL, USA), all mice variables were checked for normality assumptions. The Shapiro-Wilk-Normality test was performed across the five control and experimental mice groups. Parametric one-way ANOVA, with Bonferroni's post-hoc test, or non-parametric Kruskal-Wallis test, with Dunn's post-hoc test, were used to compare the following mice groups: sham with sens; sens with FOS, OIT and OIT+FOS; FOS with OIT+FOS; OIT with OIT+FOS. Data with regards to α-diversity (within-sample) calculated indices (richness, evenness, Simpson Index and Shannon Index) (Jost, 2007; Morris *et al.*, 2014), β-diversity (between-sample) microbial compositions (relative abundance of bacterial taxa) (Lozupone *et al.*, 2007; Mandal *et al.*, 2015) and SCFA concentrations were exported, analysed, and graphically depicted as mean ± standard error mean (SEM) with GraphPad Prism (v.7) software (GraphPad Software, La Jolla, CA, USA).

Alpha-diversity indices were calculated such as: Shannon index ($H'=-\sum \text{sum}(Pi/\log(Pi))$ where Pi = the relative abundance of each taxon), Pielou's evenness ($J'=H'/\log(S)$) where S = number of taxa present in each sample), richness (number of taxa present in each sample), and Simpson's

index (D=∑(Pi2)) where Pi = the relative abundance of each taxon.

Beta-diversity stacked histograms represent the percent relative abundance of individual taxa per mice group. Furthermore, the *Firmicutes* to *Bacteroidetes* (F/B) ratio between mice groups was studied. Correlations between SCFA concentrations (total SCFA, acetic acid, propionic acid, butyric acid and total butyric acid-to-total SCFA) and individual microbial taxa (phylum, family, and genus) were analysed by Pearson's correlation analysis. Calculated *P*-values were corrected for the number of comparisons and statistical significance was set at $P<0.05$. Additionally, PICRUSt significance was accepted at $P<0.05$ based on the experiment's hypothesis derived a priori functional pathways.

3. Results

Microbiota analysis revealed no differences in α-diversity index across the groups

Alpha-diversity represents the microbial diversity (richness and evenness) within a sample or, in this case, group of mice. The faecal microbiota analysis indicated no significant changes in α-diversity indices across the control and experimental groups at all taxonomic levels. Depicted are the results of the taxonomic level of genus (Supplementary Figure S1A-D).

Bacterial community structures in sensitised mice treated with the combination of OIT+FOS showed a high level of resemblance with non-allergic mice

Beta-diversity measures the variety in bacterial communities between samples or groups of mice. ANOSIM was performed at the taxonomic level of genus to investigate the level of similarity in bacterial community structures between the control and experiment groups (Table 1). FOS supplementation significantly impacted microbiota community structure in allergic mice. Calculated p-values indicated a significant difference (global r=0.203; $P=0.03$) in overall bacterial community structure in the allergic mice receiving the FOS supplemented diet (shown in green) compared to the allergic mice receiving the control diet (shown in red). Interestingly, a high level of similarity in bacterial community structure was observed between the sham-sensitised control group (shown in dark blue) and the OIT+FOS group (shown in light blue) (global r=0.05; $P=0.24$) (Figure 2).

OIT and/or FOS supplementation induced specific differences in relative abundance of bacterial communities

Figure 3A shows the relative abundance of the four main phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*) in the specific groups of mice. Figure 3B specifies the relative abundance distribution per group at the taxonomic level of genus. Group comparisons of the relative abundance data were analysed at all taxonomic levels and only the significantly differing phylum and genera are depicted in Figure 4. As shown in Figure 4A, the relative abundance of the putative pro-inflammatory phylum *Proteobacteria* was increased in the OIT group compared to the OIT+FOS group. The ratio of the *Firmicutes* to *Bacteroidetes* was determined, but no significant differences between the groups were observed (data not shown). An increase in relative abundance of the genus *Allobaculum* (*Firmicutes*) was observed in sensitised mice fed the FOS supplemented diet (FOS group) compared to the control diet (sens group) (Figure 4B). Three genera were shown to be different between the sensitised control mice fed the control diet and the OIT+FOS group: unclassified *Rikenellaceae* (*Bacteroidetes*, Figure 4C), unclassified *Clostridiales* (*Firmicutes*, Figure 4D) and *Oscillaspira* (*Firmicutes*, Figure 4E). In addition, the relative abundance of the putative pro-inflammatory bacterial genera *Oscillaspira* and unclassified *Rikenellaceae* were reduced in the OIT+FOS group compared to the OIT

Table 1. Across-group analysis of similarity (ANOSIM) results for mice faecal microbiota compositions.[1,2,3]

Comparisons – genus taxonomic level	n	Global r	*P*-value
(A) PBS Sens_PBS Tx_Control Diet vs (B) Whey Sens_PBS Tx_Control Diet	12	0.104	0.18
(B) Whey Sens_PBS Tx_Control Diet vs (C) Whey Sens_PBS Tx_FOS Diet	15	0.203	0.03
(B) Whey Sens_PBS Tx_Control Diet vs (D) Whey Sens_OIT 10 mg Whey_Control Diet	14	-0.007	0.46
(B) Whey Sens_PBS Tx_Control Diet vs (E) Whey Sens_OIT 10 mg Whey_FOS Diet	15	0.071	0.15
(C) Whey Sens_PBS Tx_FOS Diet vs (E) Whey Sens_OIT 10 mg Whey_FOS Diet	16	0.064	0.18
(D) Whey Sens_OIT 10 mg Whey_Control Diet vs (E) Whey Sens_OIT 10 mg Whey_FOS Diet	15	0.076	0.12

[1] Sens = sensitivity; Tx = treatment; n = number of samples.

[2] Global r comparison was based on ANOSIM performed within the software package Primer7.

[3] *P*-values were calculated based on a permutational analysis, employing 999 permutations; square-root transformation analysis.

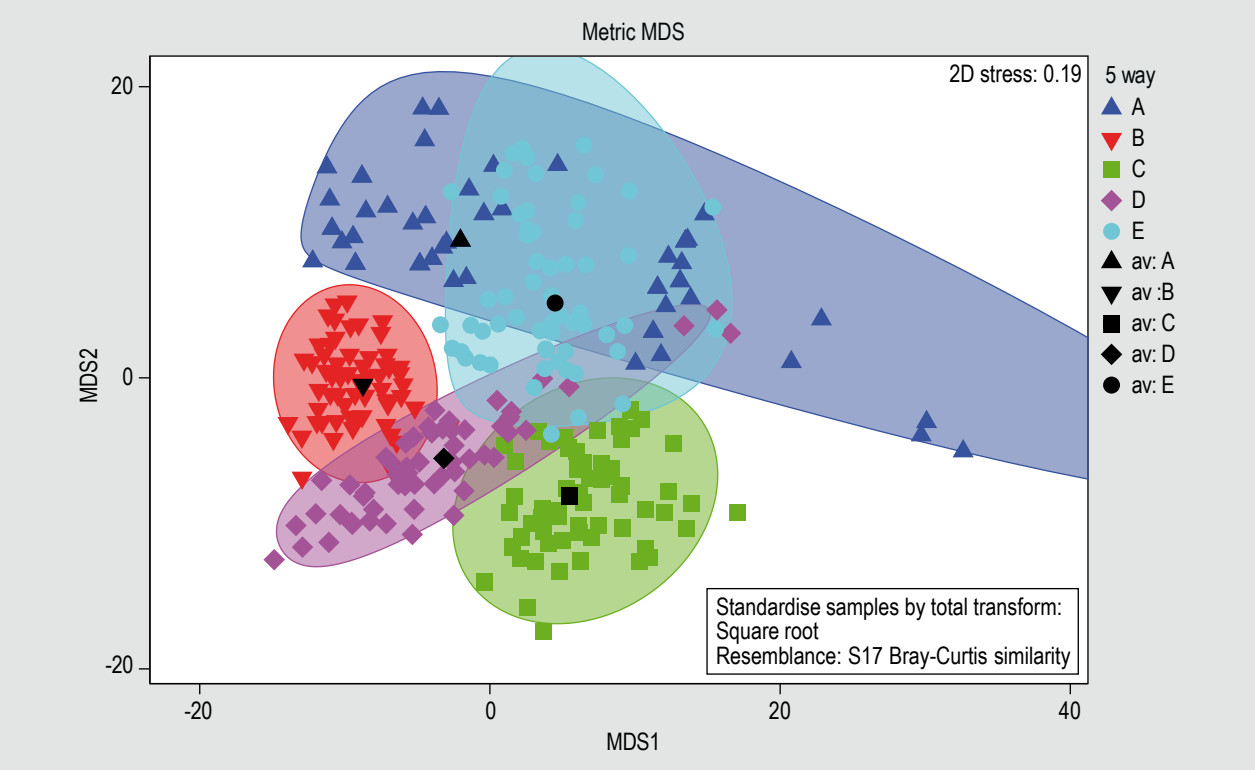

Figure 2. Overall microbial community structures in a metric multi-dimensional scaling (MDS) plot, at the taxonomic level of genus. The bacterial community structure in sensitised control mice fed the control diet (shown in red) was significantly different from sensitised mice fed the fructooligosaccharides (FOS) diet (shown in green) (ANOSIM: global r =0.203; *P*=0.03). A high level of resemblance was observed in the community structures of the sham-sensitised control mice (shown in dark blue) and the sensitised mice treated with the combination oral immunotherapy (OIT)+FOS (shown in light blue). A = sham; B = sens, C = FOS; D = OIT; E = OIT+FOS; Av = average value per group (based on Bootstrapping procedure in Primer7).

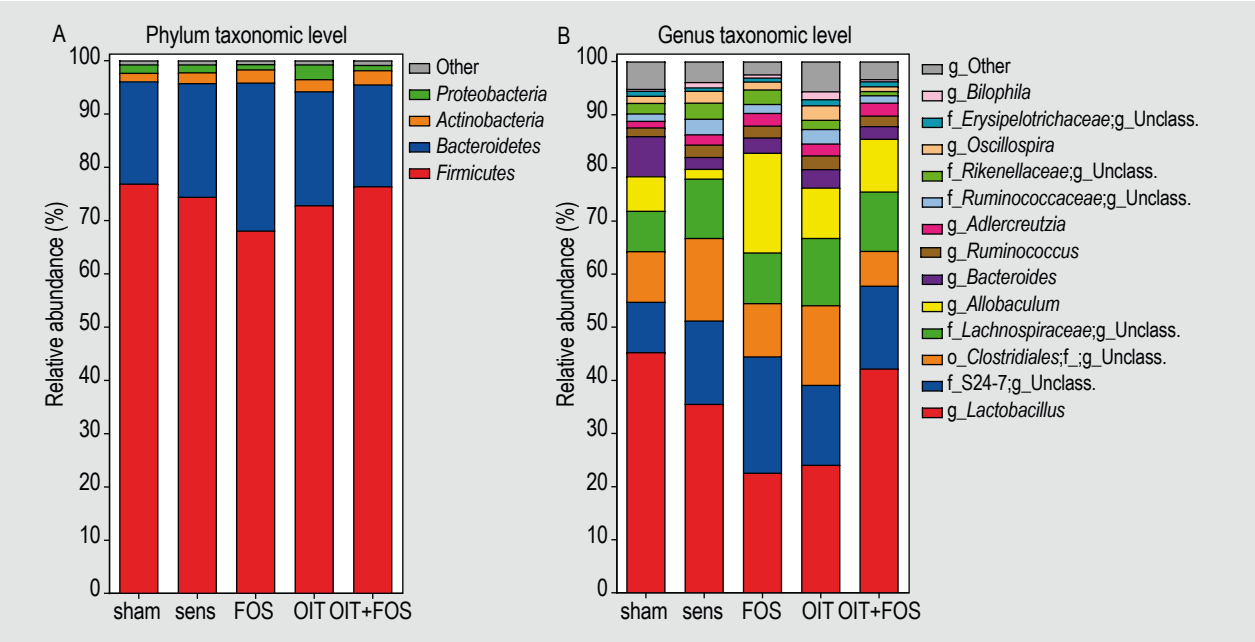

Figure 3. Stacked column plots showing the average relative abundance, at the taxonomic levels of phylum and genus. (A) Relative abundances (%) of the four main phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*) in each control and experimental group of mice. (B) Relative abundances (%) of the twelve most enriched bacterial genera in faecal samples in each control and experimental group of mice. FOS = fructooligosaccharides; OIT = oral immunotherapy.

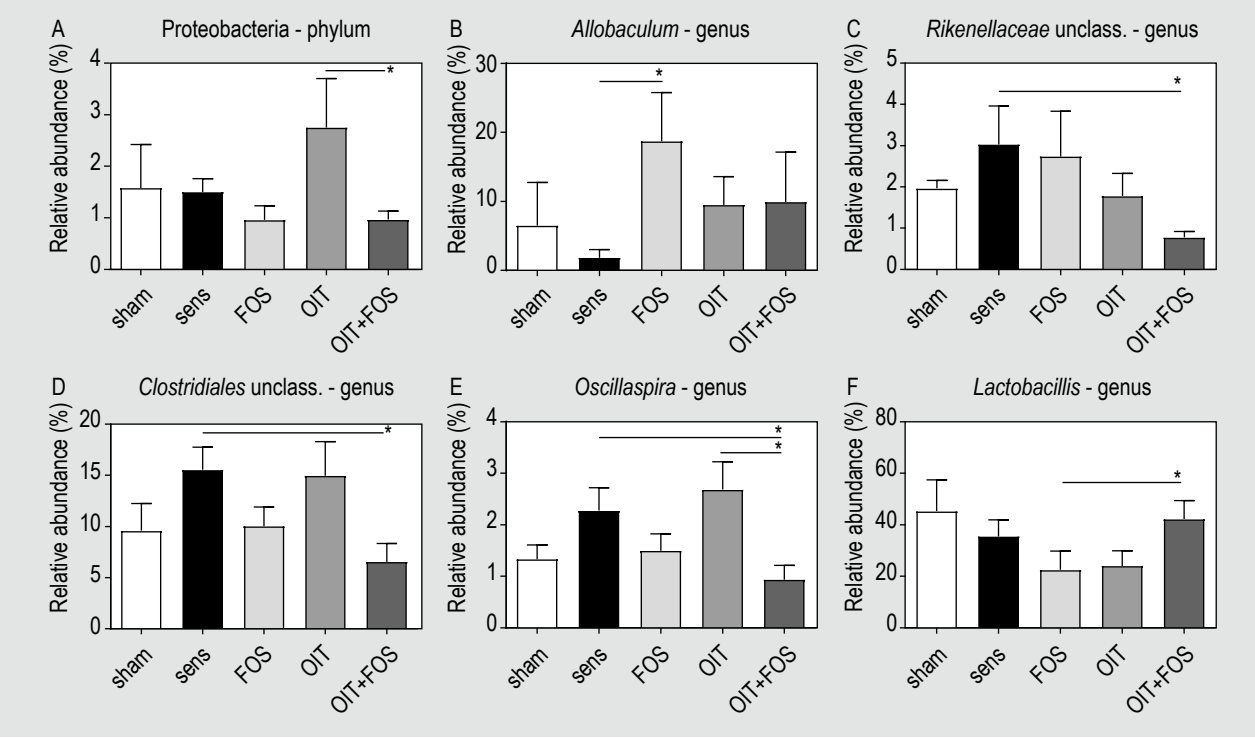

Figure 4. Differences in β-diversity in faecal samples derived from treated mice, at the taxonomic levels of phylum and genus. (A) Phylum *Proteobacteria*; (B) genus *Allobaculum* (*Firmicutes*); (C) genus unclassified *Rikenellaceae* (*Bacteroidetes*); (D) genus unclassified *Clostridiales* (*Firmicutes*); (E) genus *Oscillospira* (*Firmicutes*); (F) genus *Lactobacillus* (*Firmicutes*). FOS = fructooligosaccharides; OIT = oral immunotherapy. Data are represented as mean ± standard error of the mean, n=5-8/group. Statistical analysis was performed using Kruskal-Wallis test for non-parametric data with Dunn's post-hoc test to compare pre-selected combinations as indicated. *P<0.05.

group (Figure 4E). The genus *Lactobacillus* (*Firmicutes*) was increased in faecal samples of OIT+FOS mice compared to FOS mice, almost reaching the level measured in the non-allergic control mice (sham) as shown in Figure 4F. It is important to note that the relative abundance of the genus *Lactobacillus* is proportionately higher compared to the other genera depicted.

Predictive functional assessment showed up-regulation of metabolic pathways in allergic mice compared to non-allergic mice

Predictive assessment of the microbial community functional potential (PICRUSt analysis) was used to infer whether functional differences exist between resident microbial communities in the control and experimental groups. The level of expression of the amplified 16S rRNA subunit genes was predicted and assigned to relevant metabolic pathways. Group comparisons suggested significant differences in the predictive functional potential in a subset of metabolic pathways. We focused on the pathways which were associated with fatty acid metabolism or fiber fermentation, according to the *a priori* defined hypothesis that these pathways are involved in modulation of the allergic response by the current intervention. In Figure 5A, significant pathways are suggested to differ between the sham-sensitised controls and the sensitised controls: all pathways showed an increase in gene expression upon oral food sensitisation compared to non-allergic mice. It should be noted that the overall amount of significantly altered pathways in the PICRUSt analysis was the highest in the analysis of non-allergic vs allergic mice. Interestingly, comparison of the control diet with the FOS supplemented diet in sensitised mice suggested reduced expression of genes associated to fiber fermentation and fatty acid and retinol metabolism in FOS supplemented mice (Figure 5B). Additional group comparisons (sens vs OIT, sens vs OIT+FOS, FOS vs OIT+FOS and OIT vs OIT+FOS) showed differences in only a limited number of (non-relevant) metabolic pathways (data not shown).

OIT+FOS treated mice showed increased concentrations of butyric acid and propionic acid in caecum content

Caecum content was collected and processed to measure concentrations of the fermentation-derived bacterial metabolites SCFA and branched-chain fatty acids (BCFA) to investigate functional aspects of the microbiota. OIT+FOS treatment increased butyric acid levels compared to sensitised controls and OIT mice (Figure 6A) and a trend

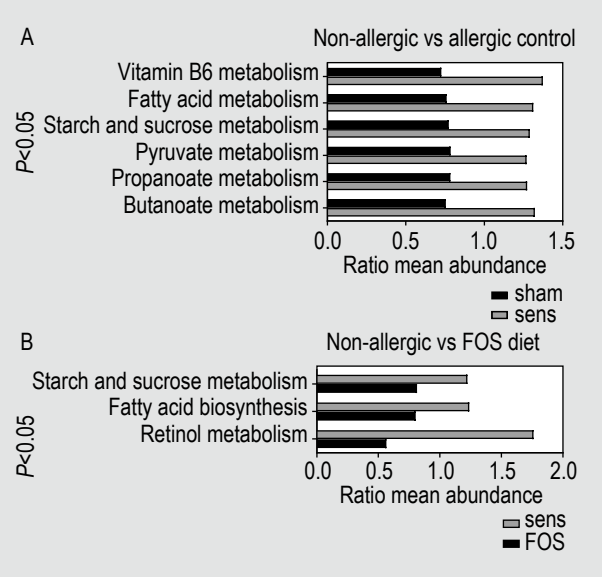

Figure 5. PICRUSt community functional predictions in relevant metabolic pathways. (A) Depicted are the inferred significantly increased metabolic pathways in whey-sensitised mice compared to sham-sensitised mice which can be linked to starch and fatty acid metabolism. (B) Significantly increased metabolic pathways in sensitised control mice compared to fructooligisaccharides (FOS) supplemented mice.

was observed compared to FOS (P=0.0504). A similar result was observed for propionic acid (Figure 6C). No differences were observed in the amount of acetic acid present in cecum content (Figure 6B). Concentrations of valeric acid were increased in OIT+FOS samples compared to the FOS group and the sensitised control group (Figure 6D). The BCFA iso-butyric acid and iso-valeric acid were increased in the OIT+FOS samples compared to the FOS samples (Figures 6E,F). However, valeric acid and BCFA levels showed a limited contribution to the total SCFA load.

Relative abundance of specific bacterial genera positively correlated to levels of propionic acid and acetic acid, but not butyric acid

Pearson correlation analysis was conducted at the taxonomic level of phylum, family and genus to investigate a potential link between the relative abundance of specific bacteria in faecal samples and the main SCFA (butyric acid, acetic acid and propionic acid) concentrations in caecum content. In Table 2, only the correlations with (P<0.05) are depicted at the taxonomic level of family and genus, since no significant correlations were observed at phylum level. The total SCFA concentration in caecum content showed a positive correlation with the abundance of the taxonomic family *Bacteroidaceae* (*Bacteroidetes*) and the following genera of bacteria: (1) *Bacteroides* (*Bacteroidetes*), (2) unclassified *Erysipelotrichaceae* (*Firmicutes*), (3) *Blautia* (*Firmicutes*), (4) unclassified *Coriobacteriaceae* (*Actinobacteria*), (5)

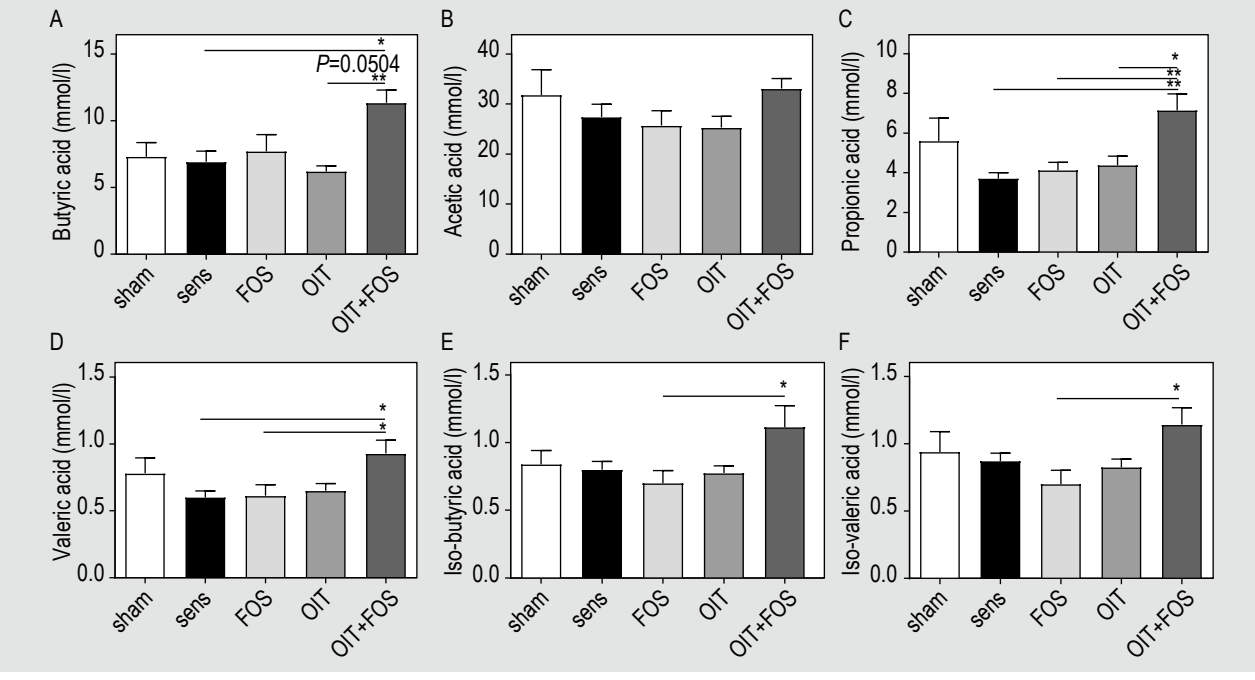

Figure 6. Short chain fatty acid and branched-chain fatty acid concentrations in caecum content of treated mice. (A) Butyric acid; (B) acetic acid; (C) propionic acid; (D) valeric acid; (E) iso-butyric acid; and (F) iso-valeric acid. FOS = fructooligosaccharides; OIT = oral immunotherapy. Data are represented as mean ± standard error of the mean, n=5-8/group. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *P<0.05, **P<0.01.

Table 2. Significant correlations comparing microbiota and short chain fatty acid (SCFA) concentrations.[1]

Taxa	SCFA r, *P*-value	Acetic acid r, *P*-value	Propionic acid r, *P*-value	Butyric acid r, *P*-value	Butyric acid/ SCFA r, *P*-value
Family					
Bacteroidaceae	+0.38, 0.02	+0.45, 0.01	+0.39, 0.02	–	–
Peptococcaceae	-0.39, 0.02	-0.34, 0.04	-0.39, 0.02	-0.36, 0.03	–
Ruminococcaceae	-0.34, 0.04	–	-0.40, 0.02	–	–
Alcaligenaceae	–	+0.36, 0.03	–	–	-0.36, 0.03
o_*Clostridiales*;f_Unclassified	–	–	-0.39, 0.02	–	–
Genus					
Bacteroides	+0.38,0.02	+0.45, 0.01	+0.39, 0.02	–	–
f_*Erysipelotrichaceae;*g_Unclassified	+0.40, 0.02	+0.47, <0.00	+0.37, 0.03	–	–
Ruminococcus	-0.37, 0.03	–	-0.40, 0.02	-0.35, 0.04	–
Blautia	+0.45, 0.01	+0.52, <0.00	+0.47, <0.00	–	–
f_*Coriobacteriaceae;*g_Unclassified	+0.44, 0.01	+0.47, <0.00	+0.53, <0.00	–	–
Proteus	+0.42, 0.01	+0.39, 0.02	+0.50, <0.00	–	–
Dorea	+0.37, 0.03	+0.43, 0.01	+0.38, 0.02	–	–
f_*Peptococcaceae*;g_Unclassified	-0.39, 0.02	-0.34, 0.04	-0.39, 0.02	-0.36, 0.03	–
Roseburia	-0.34, 0.04	–	-0.37, 0.03	-0.37, 0.03	–
Sutterella	–	+0.36, 0.03	–	–	-0.36, 0.03
o_*Clostridiales*;f_;g_Unclassified	–	–	-0.13, 0.02		
f_*Ruminococcaceae*;g_Unclassified	–	–	-0.40, 0.02	–	–
Oscillospira	–	–	-0.37, 0.03	-0.35, 0.04	–
f_*Ruminococcaceae*;Other	–	–	-0.39, 0.02	–	–
f_*Leuconostocaceae*;g_Unclassified	–	–	–	–	+0.34, 0.04
Lactococcus	–	–	–	–	+0.34, 0.04

[1] r=Pearson r-value; *P*-value=*P*<0.05; no significant differences at phylum level.

Proteus (*Proteobacteria*) and 6(() *Dorea* (*Firmicutes*) (Table 2). The relative abundance of a total of 7 bacterial genera was positively correlated to acetic acid concentrations in mice. In addition, propionic acid concentrations were positively correlated to the relative abundance of 6 bacterial genera in faecal samples. Interestingly, none of the observed correlations between either bacterial families or genera and butyric acid concentrations were positive; in fact there was a negative correlation between butyric acid levels and butyrate-producing *Roseburia*. This could potentially be due to relatively low abundance of these bacteria in the caecal content in these mice. However, the ratio of butyric acid to total SCFA levels was positively correlated to the relative abundance of two bacterial genera within the order *Lactobaccillales* (*Firmicutes*): *Lactococcus* and unclassified *Leuconostocaceae*, both lactic acid-producing bacteria. It is well known that lactic acid could be used by butyrate-producing bacteria (i.e. cross-feeding), resulting in increased production of butyric acid without increasing the abundance of butyrate-producing bacteria (Belenguer *et al.*, 2006; Louis and Flint, 2009; Riviere *et al.*, 2016).

4. Discussion

In a previous study, we showed that dietary supplementation using FOS improved OIT-induced desensitisation in cow's milk allergic mice as demonstrated by enhanced suppression of acute allergic symptoms (i.e. acute allergic skin response and mast cell degranulation) upon food challenge. The suppressed clinical response was accompanied by control of specific IgE levels, reduced Th2-mediated cytokine production in splenocytes and increased percentage of functionally active regulatory T cells (Tregs) in lymphoid organs of OIT+FOS mice (Vonk *et al.*, 2017). In the current article, we performed additional analyses on faeces and cecum content collected from these mice, to investigate potential alterations in microbial composition and metabolism, underlying the allergy suppressive effects.

Our current study indicated that 16S rRNA sequencing of microbial communities resident in faecal samples revealed no differences in microbial diversity within each group of mice (α-diversity). Evenness and richness of bacterial communities were unaltered after induction of CMA and treatment with either OIT or FOS or the combination of

both. In humans, the establishment of atopic disorders like asthma and allergies is characterised by alterations in microbial diversity and functionality in early infancy; however, there is an inconsistent association between allergy development and changes in α-diversity (Abrahamsson *et al.*, 2014; Arrieta *et al.*, 2015; Berni Canani *et al.*, 2016; Thompson-Chagoyan *et al.*, 2010). Upon analysing between-group bacterial diversity, clustering of bacterial communities in whey-sensitised mice (multi-dimensional plot) appeared to be different from the profile observed in sham-sensitised mice. However, specification of relative abundances of bacterial phyla and genera did not show significant differences between whey-sensitised and sham-sensitised mice.

A significant difference in the overall bacterial community structure was observed in sensitised mice receiving the FOS supplemented diet compared to sensitised mice receiving the control diet. In particular, the genus *Allobaculum* (family *Erysipelotrichaceae*, phylum *Firmicutes*) was increased in faecal samples of FOS supplemented mice. Furthermore, the abundance of a member of the same family of *Erysipelotrichaceae* (genus unclassified) positively correlated with the total SCFA, acetic- and propionic acid levels in cecum content of the mice. *Allobaculum* have been suggested to be putative SCFA-producers (Everard *et al.*, 2014; Greetham *et al.*, 2004; Raza *et al.*, 2017; Zhang *et al.*, 2012, 2015), and might benefit host-health via the control of inflammatory responses.

OIT+FOS treatment in cow's milk allergic mice induced a bacterial profile with a high level of resemblance with the profile observed in non-allergic mice. Differences in beta-diversity of specific genera were observed in OIT+FOS faecal samples compared to whey-sensitised control samples: OIT+FOS decreased the abundance of a member of the potentially pro-inflammatory unclassified *Rikenellaceae* (family *Rikenellaceae*, phylum *Bacteroidetes*). Food allergy-prone mice were shown to have a specific microbial signature recognised by changes in abundance of, amongst others, the family *Rikenellaceae* after sensitisation to ovalbumin compared to wild-type mice (Rivas *et al.*, 2013). Transfer of gut microbiota to germ-free wild-type mice resulted in induction of the allergic phenotype, suggesting the involvement of the family *Rikenellaceae* in the development of food allergies (Rivas *et al.*, 2013). In the current study, two genera derived from the order *Clostridiales* (phylum *Firmicutes*), *Oscillospira* (family *Ruminococcaceae*) and unclassified *Clostridiales* (family unclassified), were both significantly reduced in abundance in OIT+FOS samples compared to sensitised controls. Moreover, the relative abundance of *Oscillospira* was decreased in OIT+FOS compared to OIT. Both genera approximated the relative abundance observed in the sham-sensitised controls. Even though *Clostridiales* are recognised as the main commensals in the healthy murine colon (Nagano *et al.*, 2012) and *Oscillospira* have the ability to produce butyrate from host glycans or animal-derived sugar structures (Gophna *et al.*, 2017), our observations suggest that an increased abundance is associated with the allergic phenotype induced by sensitisation and sustained by OIT. The use of host glycans, a major component of the mucus layer in the gut, represents a potential mechanism for involvement of *Oscillospira* in promoting the allergic phenotype, since disruption of the mucus layer might result in increased intestinal permeability and activation of mucosal immune cells including mast cells. In addition, negative correlations were observed between the abundance of *Oscillospira* and butyric- and propionic acid concentrations in the mice. Our observations are potentially in contrast with previously reported data obtained from an experimental food allergy model showing a pronounced role for *Clostridiales* clusters XIVa, IV, and XVIII in dampening inflammatory and allergic responses (Atarashi *et al.*, 2013). In addition, high levels of peanut-specific and total IgE and symptoms of anaphylaxis upon challenge in sensitised germ-free mice could be abrogated in mice colonised with commensal *Clostridiales* (Stefka *et al.*, 2014). Nevertheless, the current study is the first to describe microbial changes in relation to OIT and FOS supplementation in allergic mice. To date, the opinion on *Clostridiales* with regard to favorable outcomes in food allergy studies in humans states 'mostly beneficial'; however, heterogeneity in study protocols, diagnostic techniques and dietary composition restrict clear recommendations (Huang *et al.*, 2017). In future experiments, shotgun metagenomics will be required to detect specific genus and species taxonomic annotation, to better understand microbial changes associated with unclassified *Clostridiales*, as observed in the current study.

The strategy to combine OIT and FOS supplementation to treat cow's milk allergic mice induced a specific microbial profile in faecal samples, since limited similarity in bacterial community structures was observed compared to either OIT-treated mice or FOS-treated mice. An increase in abundance of the genus *Lactobacillus* (family *Lactobacillaceae*, phylum *Firmicutes*) was observed in OIT+FOS faecal samples compared to FOS faecal samples, with levels approximating the abundance in non-allergic mice. Amongst others, *Lactobacillus* species are used as probiotics in experimental models for the prevention of food allergy or allergic asthma (Shin *et al.*, 2017; Wang *et al.*, 2017) and were shown to support natural resolution of CMA in infants fed extensively hydrolysed casein formula in a randomised trial (Canani *et al.*, 2012). Presence of *Lactobacilli* in the gastrointestinal tract is associated with host-health and reduced development of allergies (Sepp *et al.*, 1997). Interestingly, the relative abundance of the phylum *Proteobacteria* was significantly increased in faecal samples derived from mice subjected to OIT compared to OIT+FOS. With regard to the available literature concerning gut dysbiosis and *Proteobacteria* (Huang *et al.*, 2015; Keshavarzian *et al.*, 2015; Morgan *et al.*, 2012;

Shin *et al.*, 2015), we hypothesise that a causal relationship exists between OIT and the suboptimal clinical outcomes we observed in cow's milk allergic mice (Vonk *et al.*, 2017) and the increased abundance of *Proteobacteria*. One of the underlying mechanisms triggering *Proteobacteria* flares includes colonic epithelial dysfunction. Colonic epithelial cells are a main source of cellular oxygen (Rivera-Chaves *et al.*, 2016) in an otherwise anaerobic gut environment and any disruption in the amount of oxygen diffusing into the lumen can alter the anaerobiosis and thereby the growth of opportunistic facultative anaerobes, including the *Proteobacteria*, in expense of the obligate commensal anaerobes, including the *Firmicutes* and *Bacteroidetes* (Litvak *et al.*, 2017).

The predictive functionality assessment (PICRUSt) inferred that the induction of the food allergic phenotype increased bacterial-driven metabolic pathways in the gut, since the majority of up-regulated pathways occurred in whey-sensitised mice compared to the sham-sensitised mice. Additional group comparisons did not show differences in (relevant) metabolic pathways, except between whey-sensitised control mice and whey-sensitised mice supplemented with FOS. Specifically, the current data showed decreased expression of fatty acid metabolism or starch fermentation-related genes upon FOS supplementation in sensitised mice compared to sensitised mice fed a control diet. The latter observation was in contrast to the *a priori* defined hypothesis that FOS supplementation would increase gene expression related to bacterial fiber fermentation. This unexpected observation raises the question whether prebiotic fibers might help to restore a balanced state of the bacterial metabolome, rather than stimulating metabolic processes. It has been described that resolution of CMA was associated with decreased expression of genes involved in fatty acid metabolism by the gut microbiome (Bunyavanich *et al.*, 2016). Nevertheless, PICRUSt analysis indicated that the effect of prebiotic fiber supplementation on the bacterial metabolome might be different in an allergy skewed gut environment. Future studies using shotgun metagenomics are required to further investigate the results of PICRUSt analysis.

Together, the composition of the gut microbiome and fermentation of both carbohydrates and dietary proteins determine the availability of specific bio-active compounds (e.g. SCFA) in local and peripheral tissues via uptake in the systemic circulation (Windey *et al.*, 2012; Wypych and Marsland, 2017). In the current study, SCFA concentrations in cecum content were elevated in mice treated with the combination OIT+FOS, rather than FOS supplementation or OIT alone. We questioned whether the altered (allergic) immune response affected bacterial metabolic activity and thereby the availability of SCFA. The elevated levels of butyric acid, as observed in OIT+FOS mice only, have potentially contributed to the reduced allergic symptoms and suppressed mast cell degranulation upon food challenge (Vonk *et al.*, 2017). Supplementation with butyric acid, but not propionic acid, via the drinking water in peanut allergic mice was previously shown to effectively reduce anaphylaxis symptoms (Tan *et al.*, 2016). In addition, oral butyrate supplementation was as effective as FOS supplementation in supporting desensitisation of cow's milk allergic mice induced by OIT (Vonk *et al.*, 2019). Interestingly, correlation analyses revealed that none of the bacterial communities detected in murine faecal samples were positively correlated to butyric acid levels. This observation highlights that caution is warranted when discussing the source of the elevated butyric acid levels. Increased butyric acid concentrations could originate from indirect fermentative reactions that convert acetic acid into butyric acid (Louis *et al.*, 2007). Furthermore, a positive correlation was observed with the relative abundance of lactic acid-producing bacteria and the butyric acid/SCFA ratio. This observation suggests another indirect mechanism of butyric acid production that could be involved, since lactic acid can be converted into butyric acid by members of the *Clostridiales* cluster (*Firmicutes*) (Louis and Flint, 2009). It remains speculative whether OIT+FOS treatment in cow's milk allergic mice stimulates the activity of bacteria that either produce acetic acid and lactic acid, and/or convert acetic acid and lactic acid, rather than butyrate-producing bacteria. Future studies should include in-depth analysis of a broad range of metabolites to link alterations in the bacterial composition and metabolic activity to improved allergic outcomes in a food allergy setting.

5. Conclusions

The combination of OIT+FOS to treat cow's milk allergic mice altered microbial diversity and metabolism and induced a microbial profile closely linked to non-allergic control mice. Improved treatment efficacy of OIT by FOS supplementation was previously only shown by reduced allergic symptoms upon food provocation, and the current study contributes to our understanding of the involvement of the gut microbiota. Further research, using metagenomics, will be required to better understand the composition of the gut bacteria and it's functional/mechanistic roles.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2019.0024.

Figure S1. Alpha-diversity scores in faecal samples derived from treated mice, at the taxonomic level of genus. (A) richness, (B) evenness, (C) Shannon Index and (D) Simpson Index.

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Conflict of interest statement

None of the authors have a competing financial interest in relation to the presented work; Dr. L.M.J. Knippels is employed, and Dr. B.C.A.M. van Esch and Prof. dr. J. Garssen are partly employed by Danone Nutricia Research, Utrecht, the Netherlands.

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