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Mice co-administrated with partially hydrolysed whey proteins and prebiotic fibre mixtures show allergen-specific tolerance and a modulated gut microbiota

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RESEARCH ARTICLE

Abstract

Non-breastfed infants at-risk of allergy are recommended to use a hydrolysed formula before the age of 6 months. The addition of prebiotics to this formula may reduce the allergy development in these infants, but clinical evidence is still inconclusive. This study evaluates (1) whether the exposure duration to different prebiotics alongside a partially hydrolysed whey protein (pHP) influences its' effectiveness to prevent allergy development and (2) whether the gut microbiota plays a role in this process. Mice orally sensitised with whey and/or cholera toxin were orally treated for six days before sensitization with phosphate buffered saline, whey or pHP to potentially induce tolerance. Two groups received an oligosaccharide diet only from day -7 until -2 (GFshort and GFAshort) whereas two other groups received their diets from day -15 until 37 (GFlong and GFAlong). On day 35, mice underwent an intradermal whey challenge, and the acute allergic skin response, shock score, and body temperatures were measured. At day 37, mice received whey orally and serum mouse mast cell protease-1, SLPI and whey-specific antibodies were assessed. Faecal samples were taken at day -15, -8 and 34. Feeding mice pHP alone during tolerance induction did not reduce ear swelling. The tolerance inducing mechanisms seem to vary according to the oligosaccharide-composition. GFshort, GFlong, and GFAlong reduced the allergic skin response, whereas GFAshort was not potent enough. However, in the treatment groups, the dominant *Lactobacillus* species decreased, being replaced by *Bacteroidales* family S24-7 members. In addition, the relative abundance of *Prevotella* was significantly higher in the GFlong, GFAshort and GFAlong groups. Co-administration of oligosaccharides and pHP can induce immunological tolerance in mice, although tolerance induction was strongest in the animals that were fed oligosaccharides during the entire protocol. Some microbial changes coincided with tolerance induction, however, a specific mechanism could not be determined based on these data.

Keywords: cow's milk allergy, non-digestible oligosaccharides, preventive tolerance induction, microbiota

1. Introduction

Infants at risk of allergy development that are not breastfed are recommended to use a hydrolysed formula when the formula will be introduced before the age of 6 months. Although clinical evidence is inconclusive, it has been suggested that the addition of specific oligosaccharides may reduce the allergy susceptibility. Boyle *et al.* (2016) showed that a partially hydrolysed formula containing a specific mixture of oligosaccharides had no positive effect on eczema incidence at 18 months or total/specific IgE at 6 months. However, they did observe lower levels of cow's milk-specific IgG1 (a marker of sensitization/immunization) and increased numbers of regulatory T cells (Treg) and plasmatoid dendritic cells (DCs), suggestively providing a tolerogenic environment (Boyle *et al.*, 2016).

Tolerance is the default response of the immune system in healthy individuals to novel harmless protein antigens. Mucosal DCs are pivotal in the maintenance of this tolerance that, in turn, is essential to secure intestinal immune homeostasis. The mucosal DCs enable the mucosal immune system to generate a protective inflammatory response against potential pathogens while remaining quiescent when harmless proteins such as food antigens enter the intestine. A specific subpopulation of DCs in the intestines, the plasmatoid CD103+ DC, is thought to be involved in tolerance induction because of their role in the local generation of regulatory T-cells (Tregs), including Foxp3+ Tregs (Coombes *et al.*, 2007; Iwasaki, 2007; Jaensson *et al.*, 2008). Our previous murine studies have shown similar findings: Tregs and CD4+CD69+CXCR3+Th1 cells (Schouten *et al.*, 2010, 2012) are important cell types that along with increased levels of transforming growth factor-beta and interleukin 10 (Kerperien *et al.*, 2014) are essential in building oral tolerance towards cow's milk proteins. In addition, multiple studies have shown that the used prebiotic mixtures of short chain galacto-oligosaccharides (scGOS) and long chain fructo-oligosaccharides (lcFOS) (scGOS:lcFOS (GF) in a ratio 9:1) or scGOS, lcFOS and pectin-derived acidic oligosaccharide (pAOS) (scGOS:lcFOS:pAOS (GFA) in a ratio 9:1:2) create an intestinal environment that promotes immunological tolerance induction (Kerperien *et al.*, 2014; Schouten *et al.*, 2010, 2012).

Accumulating data suggest that early-life microbiome composition is associated with childhood allergic asthma (reviewed in Fujimura and Lynch, 2015; Lynch, 2016) These findings provide a rationale to consider microbiota manipulating in order to prevent or treat the development of allergy. In addition to the direct effects of prebiotics on the immune response development, prebiotics also play a crucial role in gut microbiota development in early life. The development towards a relatively stable composition as observed in adults is dynamic and can be influenced by environmental factors such as early life nutrition and delivery mode (Wopereis *et al.*, 2014). There are strong indications that prebiotic modulation of the microbiota can lead to reduced incidence of atopic disease. In a trial in Italian infants at risk of developing atopy, supplementing hydrolysed formula with 9:1 scGOS:lcFOS led to a reduction in atopic dermatitis, associated with increased number of *Bifidobacterium* (Moro *et al.*, 2006) Similarly, allergy and infections were reduced in a comparable study using the same prebiotic mixture to supplement hypoallergenic formula during the first six months of life (Arslanoglu *et al.*, 2008). Further insights in the potential link between gut microbiota development and mucosal immune tolerance development through exposure to specific prebiotic mixtures and/or (hydrolysed) proteins will help to design novel food concepts to prevent the development of allergic responses in early life. The objective of this study was to evaluate whether the partially hydrolysed whey exposure in combination with different prebiotic mixtures influences the effectiveness of allergy prevention in a mouse model for cow's milk allergy. In addition, the potential role of the gut microbial development within this immunological tolerance induction was investigated. To address the importance of timing of prebiotic supplementation, the different prebiotic mixtures were given during tolerance induction or starting at day -15 until the end of the experiment. Previous data suggests different mechanisms of action between GF and GFA on reduction of allergic symptoms in a mouse model for cow's milk allergy (Kerperien *et al.*, 2014). In this study we will investigate the effect of GF and GFA on microbial development as a potential mechanism of action in allergy reduction or prevention. The results of this study further strengthen our knowledge on the effectiveness of the combination of allergen-specific tolerance induction with a partial whey hydrolysate and generic immunomodulation through prebiotics.

2. Materials and methods

Cow's milk proteins and partially hydrolysed whey protein

Whey protein concentrate 80 (WPC80; Friesland Campina, the Netherlands) and demineralised whey (Deminal; Friesland Campina, Amersfoort, the Netherlands) were mixed in a ratio of 3:1, to be subsequently hydrolysed with an established mixture of endopeptidases and exopeptidases (confidential enzyme composition used by Nutricia Research, Utrecht, the Netherlands) resulting in partially hydrolysed whey proteins (pHP). The enzymatic process was stopped by fast cooling the batch to below 10 °C via a heat exchanger with cold water as cooling agent within 60 min. The pHP was characterised by the analysing the peptide size (85% <1 kD, 8% <2 kD, 4% <5 kD, 1% <10 kD, 0.6% <20 kD and 1.4% >20kD) by means of high pressure liquid chromatography. This pHP and a non-hydrolysed mixture of WPC80 plus Deminal (ratio 3:1) (collectively called 'whey') were used for the experiments described below.

Diet

Semi-synthetic cow's milk-protein free (milk proteins are replaced by soy protein) AIN-93G-based diets were composed and mixed with non-digestible oligosaccharides by Ssniff Spezialdiäten GmbH (Soest, Germany). Carbohydrates in Vivinal® GOS (Friesland Campina) were compensated isocalorically in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose) and dextrose (for glucose). In case of the fructo-oligosaccharides, carbohydrates were compensated isocalorically in the control diet by means of exchange against cellulose (for FOS). Dietary compositions were

composed of the cow's milk free AIN-93G diet (CTR diet), scGOS/lcFOS (9:1; 0.8 w/w%) (GF diet) and scGOS/lcFOS (9:1; 0.8 w/w%) plus pAOS (2; 0.2 w/w%) (GFA diet), and the diets were stored at -20 °C. The diets were completely refreshed every week. The mice had *ad libitum* access to their respective diets and water.

Mouse model

The care and use of animals was performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Four-week-old specific pathogen-free female C3H/HeOuJ mice that were at least two generations on cow's milk free diet and weighed at least 11 g were purchased from Charles River Laboratories (Erkrath, Germany). Per treatment, mice were housed per 5 animals in a Makrolon type III^{low} cage. Mice were randomly assigned to the cages, and each cage was randomly allocated to the different treatments. Two cages were allocated to each treatment, adding up to 10 animals. The husbandry conditions were set on 12/12 h light/dark cycle, 22.3±0.6 °C and 65.3±3.0% humidity. As nesting material, the wood saw dust laboratory bedding Lignocel 9S (Tecnilab-BMI, Someren, the Netherlands) was used, and two red-transparent polycarbonate cages were added to each cage as environmental enrichment.

Mice within the GFlong and GFAlong groups started their respective diets at the day of arrival (day -15) and continued the diet throughout the whole study. This is indicated as 'prebiotic exposure throughout the study' in Figure 1. The GFshort and GFAshort groups only received their respective diet during the six oral tolerance induction gavage days (day -7 until day 0). All other days, they received the CTR diet. This is indicated as 'prebiotic exposure during Tolerance Induction' in Figure 1.

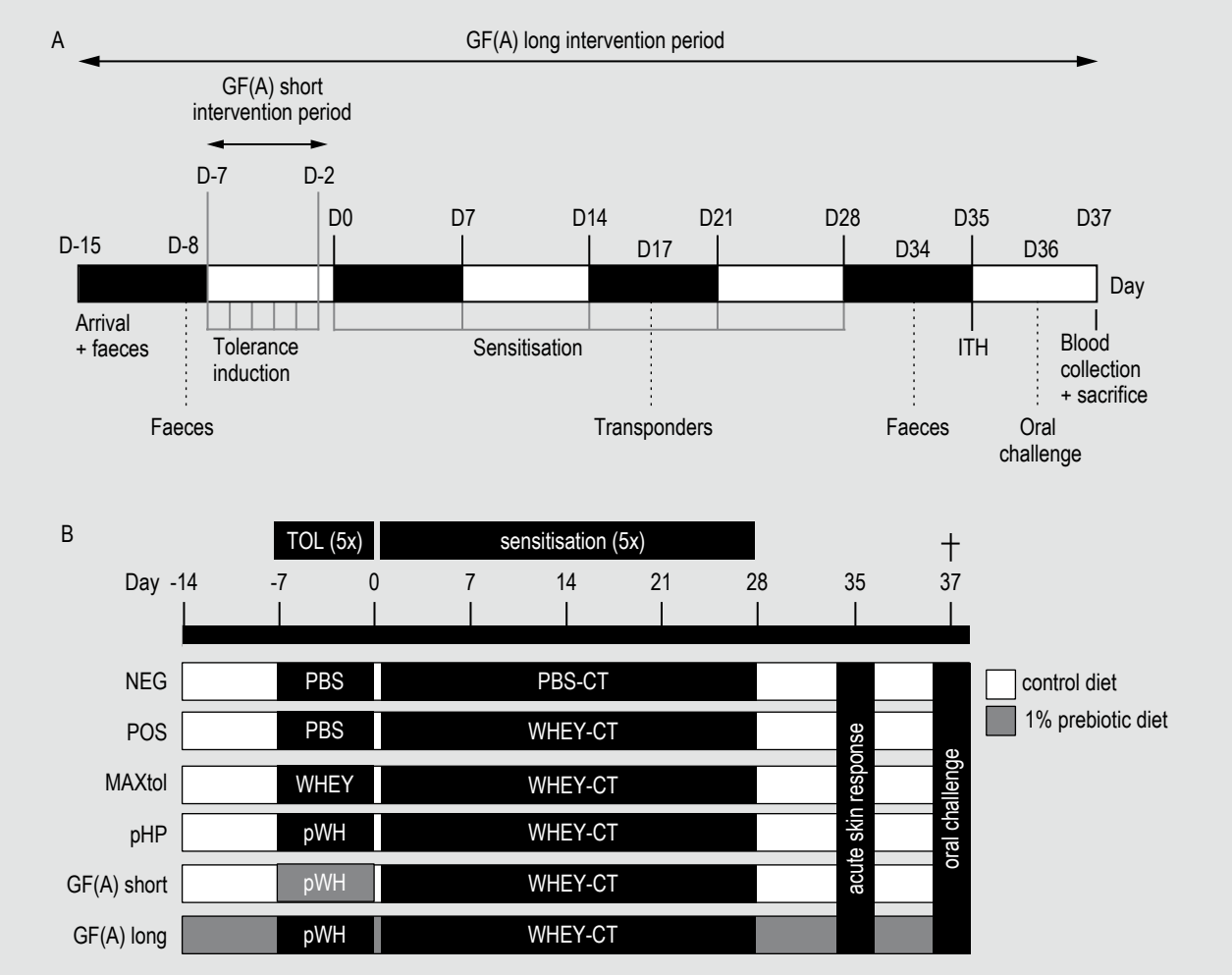

Figure 1. Schematic overview study design. D = day; GF(A) = scGOS/lcFOS or scGOS/lcFOS/pAOS intervention (mice); scGOS = short chain galacto-oligosaccharides; lcFOS = long chain fructo-oligosaccharides; pAOS = pectin-derived acidic oligosaccharide; ITH = immediate type hypersensitivity; TOL = tolerance induction; PBS = phosphate buffered saline; CT = cholera toxin; pWH = partial whey hydrolysate; WHEY = whey mixture; POS = positive control mice; NEG = negative control mice; MAXtol = maximum tolerance mice; pHP = partially hydrolysed whey protein mice.

From day -7 until day -2, mice were orally exposed to 500 µl phosphate buffered saline (PBS) (NEG and POS groups), 500 µl of 100 µg/ml whey (MAXtol) or 500 µl of 100 µg/ml pHP (all other groups) in PBS using a blunt ended stainless-steel feeding needle as reflected by the 'Tolerance Induction' period in Figure 1. Subsequently, mice were orally sensitised by gavage, on day 0, 7, 14, 21 and 28 with 500 µl of 40 mg/ml whey homogenised in PBS per animal mixed with 20 µg/ml cholera toxin (CT; Quadratech Diagnostics, Epsom, UK; #100B) as an adjuvant. The NEG mice received cholera toxin in PBS only. Between 3rd and 4th sensitisation, implantable temperature transponders (Bio Medic Data Systems, Seaford, DE, USA) were placed under isoflurane/gas anaesthesia.

Acute allergic skin response

At day 35, the immediate type hypersensitivity (ITH) was assessed in a blinded way by subtracting the ear thickness of both ears before from the thickness measured 1 h after an intradermal (i.d.) whey exposure of 20 µl of 300 µg/ml whey in PBS per ear. The ear thickness was measured in duplicate using a digital micrometre (Mitutoyo, Veenendaal, the Netherlands). The ear swelling is expressed as delta µm. Furthermore, the anaphylactic shock score according to the method described by Van Esch *et al.* (2011a) and body temperature were assessed every 15 min following the i.d. whey challenge.

Measurement of serum specific antibodies and mMCP-1

At day 36, the mice were orally exposed to 500 µl whey solution (100 mg/ml PBS) and, 16 h later (day 37), blood samples were collected by eye-extraction under isoflurane-air gas anaesthesia, followed by termination through cervical dislocation. The blood samples were centrifuged for 15 min at 20,000×*g* and serum was stored at -20 °C until further analyses including mMCP-1, whey-specific immunoglobulin (Ig)E, whey-IgG1, and whey-IgG2a. The whey-specific immunoglobulin levels were measured in serum by means of ELISA. Costar high binding Assay Plates (Corning, Tewksbury, MA, USA; #9018) were coated with 20 µg of whey in coating buffer for 18 h at 4 °C. Plates were washed and blocked for 1 h with PBS with 0.05% Tween and 1.5% Human Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA; #A1887). The murine serum samples were incubated for 2 h at room temperature. Plates were washed and incubated with 1 µg biotin-labelled rat anti-mouse IgE (Beckton Dickinson, Franklin Lakes, NJ, USA; #553419), IgG1 (Beckton Dickinson; #553441) or IgG2a (Beckton Dickinson; #553388) for 1.5 h at room temperature. After washing, the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands; #M2051) for 1 h, washed and developed with 1-Step Ultra TMB-ELISA substrate solution (Thermo Scientific, Waltham, MA, USA; #34028). The reaction was stopped after 10 min with 1 M H_2SO_4 (Fluka Analytical, Honeywell, Seelze, Germany; #35276) and absorbance was measured at 450 nm on a Benchmark microplate reader (Biotek, Winooski, VT, USA). Results were analysed using the Gen5 software (Biotek), and are expressed as arbitrary units (AU). Serum concentrations of mouse mast cell protease-1 (mMCP-1) as a reflection of local mast cell degranulation were determined according to the manufacturer's protocol using a commercially available ELISA kit (eBioscience, San Diego, CA, USA; #88-7503-88).

Faecal samples

Faecal samples were taken at arrival (day -15) and before the tolerance induction (day -8) in order to assess the microbial composition alterations due to the new housing conditions and the dietary interventions in the GF-long and GFA-long groups. An additional faecal sample was taken at day 34, to assess the changes induced by the tolerance induction and sensitization.

DNA isolation from faecal samples

Samples were weighed (average 38.7±17.2 g) and prepared for isolation using a standard bead-beating protocol (low scale bead-beating: 25 g 0.1 mm zirconia beads plus 3 glass beads in STAR buffer from Roche). DNA was extracted and purified with the Maxwell MDx (Promega Benelux B.V., Leiden the Netherlands), using the Maxwell 16 Tissue LEV Total RNA Purification Kit. DNA quality and quantity was assessed with the NanoDrop 2000 spectrophotometer (Thermo Scientific). Samples were diluted to the same concentration before subsequent processing.

Bacterial 16S rRNA gene amplicon sequencing

Samples were prepared for Illumina Miseq sequencing (Illumina, San Diego, CA, USA) using a two-step protocol for amplification of the 16s rRNA genes and barcoding of the samples. The protocol was adapted from Tian *et al.* (2016). Bacterial 16S rRNA gene fragments were amplified using universal primers covering the V3-V4 region of the bacterial 16S rRNA gene. The forward primer consisted of the S-D-Bact-0341-b-S-17 primer (5'-CCTACGGGNGGCWGCAG-3') (Klindworth *et al.*, 2013) added to the 3' end of the Unitag1 barcoding adapter (5'-GAGCCGTAGCCAGTCTGC-3'). The reverse primer consisted of the S-D-Bact-0785-a-A-21 primer (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013) added to the 3'-end of the Unitag2 barcoding adapter (5'-GCCGTGACCGTGACATCG-3').

The PCR was performed in a volume of 50 µl, containing 10 µl of 5× HF green buffer (Finnzymes, Espoo, Finland), 1 µl dNTP mix (Promega), 0.5 µl of Phusion Hot Start II DNA polymerase (2 U/µl; Finnzymes), 2.5 µl of the reverse

primer mix and the forward primer (both 10 µM), 1 µl template, and 32.5 µl nuclease free water. The PCR program was: 98 °C for 30 s to activate the enzyme, then 25 cycles of 98 °C for 10 s, 56 °C for 20 s and 72 °C for 20 s, followed by a final extension at 72 °C for 10 min. 5 µl of PCR product was used in a second PCR with 8 nucleotide sample specific barcodes, which were added to the Unitag1 and Unitag2 sequences. This second PCR mixture contained 20 µl HF green buffer (Finnzymes), 2 µl dNTP mix (Promega), 1 µl Phusion Hot Start II DNA polymerase (Finnzymes), 62 µl nuclease free water, 5 µl forward barcoded Unitag1 primer (10 µM), 5 µl reverse barcoded Unitag2 primer (10 µM) and 5 µl product of the first PCR. The PCR program started with an activation step at 98 °C for 30 s, followed by 5 cycles of 98 °C for 10 s, 52 °C for 20 s and 72 °C for 20 s, finishing with an extension step at 72 °C for 10 min. The PCR product was purified using the Highprep PCR clean-up magnetic beads (Magbio, Theale, UK). Finally, the samples were pooled in equimolar concentration, 48 samples per library (including 2 mock communities as an internal standard), after which the libraries were concentrated with the Highprep PCR beads (Magbio). The samples were analysed on the Illumina MiSeq sequencing platform.

Data analysis and statistics

For the analysis of the 16S rRNA gene sequencing data, the in-house NG-Tax pipeline was used (Ramiro-Garcia *et al.*, 2016). Briefly, paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and these barcodes were used for demultiplexing. Operational Taxonomic Unit (OTU) picking were performed with an open reference approach and a customised SILVA 16S rRNA gene reference database (Quast *et al.*, 2013). ClustalW was used to generate an alignment of OTU sequences and a corresponding dendrogram. Alpha and beta diversity metrics were calculated using scripts from the Quantitative Insights Into Microbial Ecology (QIIME) v1.8.0 package. Ordination analyses were performed with the Canoco 5.0 software package (Ter Braak *et al.*, 2012).

The *in vivo* and *in-vitro* data were analysed using D'Agostino & Pearson normality test. Data were analysed using one way ANOVA and post-hoc Dunnett's test if the values were normally distributed otherwise, data were analysed using Kruskal-Wallis and post-hoc Dunn's test. Grubbs' Test for Outliers was used to identify outliers, outliers were excluded from analysis where indicated. After dropouts (anaphylaxis), the total number of animals per group of which the data were used for statistical analysis was: n=10 non-sensitised (NEG) mice, n=7 sensitised control (POS) mice, n=10 sensitised mice receiving whole protein (MAXtol), n=10 sensitised mice receiving partially hydrolysed whey proteins (pHP), n=10 pHP mice receiving GF for a short period (GFshort), n=10 pHP mice receiving GFA for a short period (GFAshort), n=8 pHP mice receiving GF for a long period (GFlong) and n=9 pHP mice receiving GFA for a long period (GFAlong). A probability value of $P<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism 7.0 (La Jolla, CA, USA) and IBM SPSS Statistics software (Armonk, NY, USA).

3. Results

To study whether the different prebiotic mixtures combined with a pHP can induce a preventive immunological tolerance response, mice were exposed to GF or GFA together with pHP prior to sensitization. As the prebiotic mixtures can be provided either only during exposure to the pHP or throughout the study, we here compared a short to a long prebiotic intervention period (Figure 1).

Clinical parameters within the preventive tolerance induction model

As shown in Figure 2A, sensitised mice that were fed the control diet (POS) displayed a significantly higher ear swelling at 1 h (109.4±13.8 µm) compared to non-sensitised (NEG) mice (28.6±3.3 µm) after an ear challenge with whey. Preventive administration of the whole protein (MAXtol) or pHP alone induced a significantly lower acute ear swelling response (27.78±2.3 µm and 83.23±8.3 µm, respectively) compared to POS mice. A short intervention with pHP and GF induced a significantly lower acute ear swelling response (72.08±4.7 µm) compared to POS mice, whereas a short treatment with pHP and GFA did not (85.58±6.3 µm). Interestingly, both combinations of pHP with GF and GFA induced a significantly lower acute ear swelling response compared to POS mice when administered throughout the entire study protocol (GFlong: 67.44±6.4 µm; GFAlong: 70.56±6.2 µm). These results indicate that either long or short term exposure to a combination of a pHP and different prebiotic mixtures can successfully prevent the onset of acute allergic skin responses.

After oral challenge, three mice of the POS group, two mice of the GFlong group and one mouse of the GFAlong group reached the humane endpoint based on shock score and were sacrificed. Samples from these animals were excluded from further analysis. As observed previously (Van Esch *et al.*, 2011a), there was a correlation between anaphylactic shock score (Figure 2B) and the drop in body temperature 30 min after the i.d. challenge (Figure 2C). In both parameters, the POS control group was significantly different from the NEG animals, and preventive administration of whole protein was able to completely abolish this effect. However, results after preventive administration of pHP alone were not significantly different from the POS group. Only when pHP was combined with a short intervention with GF, both shock score as well as body temperature were significantly different from that seen in the POS mice. As these parameters are not completely in line with the ear

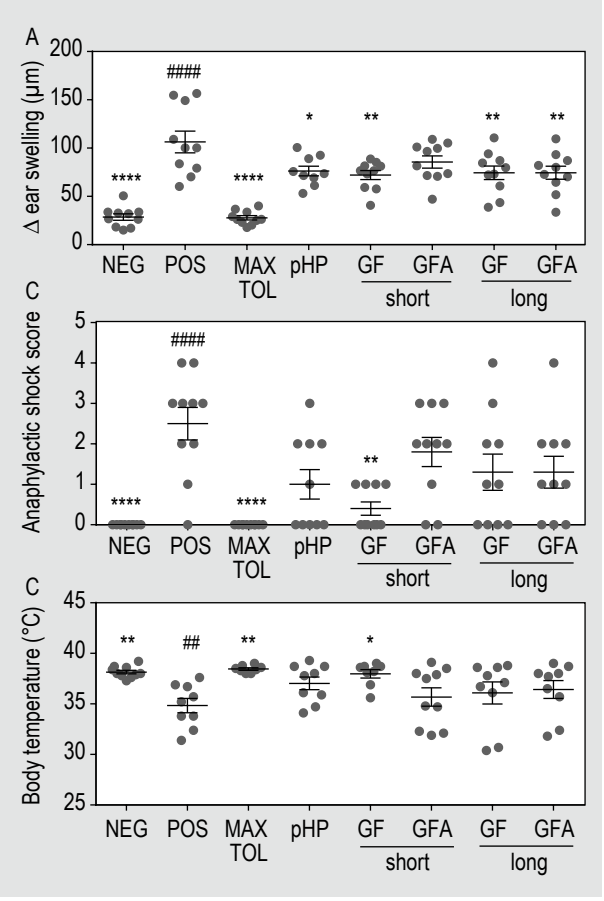

Figure 2. Clinical immune parameters at day 35. (A) Acute allergic skin response in the ears 1 h after i.d. whey challenge. One outlier was removed from analysis (pHP n=9). (B) Anaphylactic shock score 1 h after i.d. whey-challenge. (C) Body temperature 30 min after i.d. whey challenge (point of maximum drop in temperature). Several mice received a non-functional transponder and one outlier was removed from analysis (MAXtol, n=8; pHP, n=9; GFshort, n=8). Data are expressed as individual values with the group-mean ± standard error of the mean; ****P*<0.05, *****P*<0.01, *******P*<0.0001 compared to the POS group, and # *P*<0.05, #### *P*<0.0001 compared to the NEG group. For treatment abbreviations see Figure 1.

swelling outcomes, we speculate that the combination of prebiotics and pHP does not reduce the ear swelling response solely through systemic IgE-mediated mast cell degranulation.

Allergy markers in serum

In sensitized mice fed the control diet (POS), whey-specific IgE (Figure 3A), whey-IgG1 (Figure 3B) and whey-IgG2a (Figure 3C) were increased in serum when compared to non-sensitised (NEG) mice. Interestingly, all three whey-specific immunoglobulins were significantly reduced in the mice that received a preventive administration of the whole protein prior to sensitization. No reducing effects on these allergen-specific immunoglobulins were observed when the mice were treated with the pHP alone or in combination with the different prebiotic mixtures. These findings again support the hypothesis that the combination of prebiotics and pHP does not reduce the ear swelling response solely through a route that is mediated by allergen-specific immunoglobulins.

To assess whether the combination of pHP and different prebiotic mixtures affect intestinal mast cell activation, mMCP-1 serum concentrations were determined. In sensitised mice fed the control diet (POS), the mMCP-1 levels were increased in serum when compared to non-sensitised (NEG) mice, however, this did not reach significance (Figure 3D). Although not seen with preventive administration of the whole protein, administration of pHP alone showed reduced mMCP-1 levels compared to sensitised mice on a control diet (POS). When pHP was combined with the prebiotic mixtures, the reduction of the mast cell activation was only abrogated in combination with GFAlong. Thymic stromal lymphopoietin (TSLP) is involved in the pathogenesis of allergic inflammation in the lung and skin, but is also contributing to allergic inflammation in the gut as it amplifies Th2 responses (Ziegler, 2010). In sensitized mice fed the control diet (POS), TSLP levels were increased in serum when compared to non-sensitised (NEG) mice (Figure 3D). In contrast to the mMCP-1 levels, TSLP was significantly lower after the preventive administration of the whole protein, whereas none of the pHP administration groups altered TSLP levels. Together, these results indicate that TSLP levels correlate negatively with tolerance induction.

Microbial diversity

As described above, none of the determined immune parameters fully explain the mechanism through which the co-administration of pHP and the different prebiotic mixtures lead to the reduced allergic skin response. As prebiotics are known to modulate the microbial composition, the potential differences between a long and short prebiotic exposure as well as the difference between mixtures of two or three prebiotics have been studied.

When comparing the faecal samples before (day -15) and after (day -8) the run-in period, we showed that the murine microbiota composition changed significantly due to the new environment in the new animal facility (Figure 4A). Based on the hypothesis that the starting microbial composition of mice is essential within murine immune models, this finding emphasises the importance of a run-in period.

The average phylogenetic diversity of the gut microbiota of animals that did not yet undergo any immunological or dietary treatment increased significantly over time (Figure

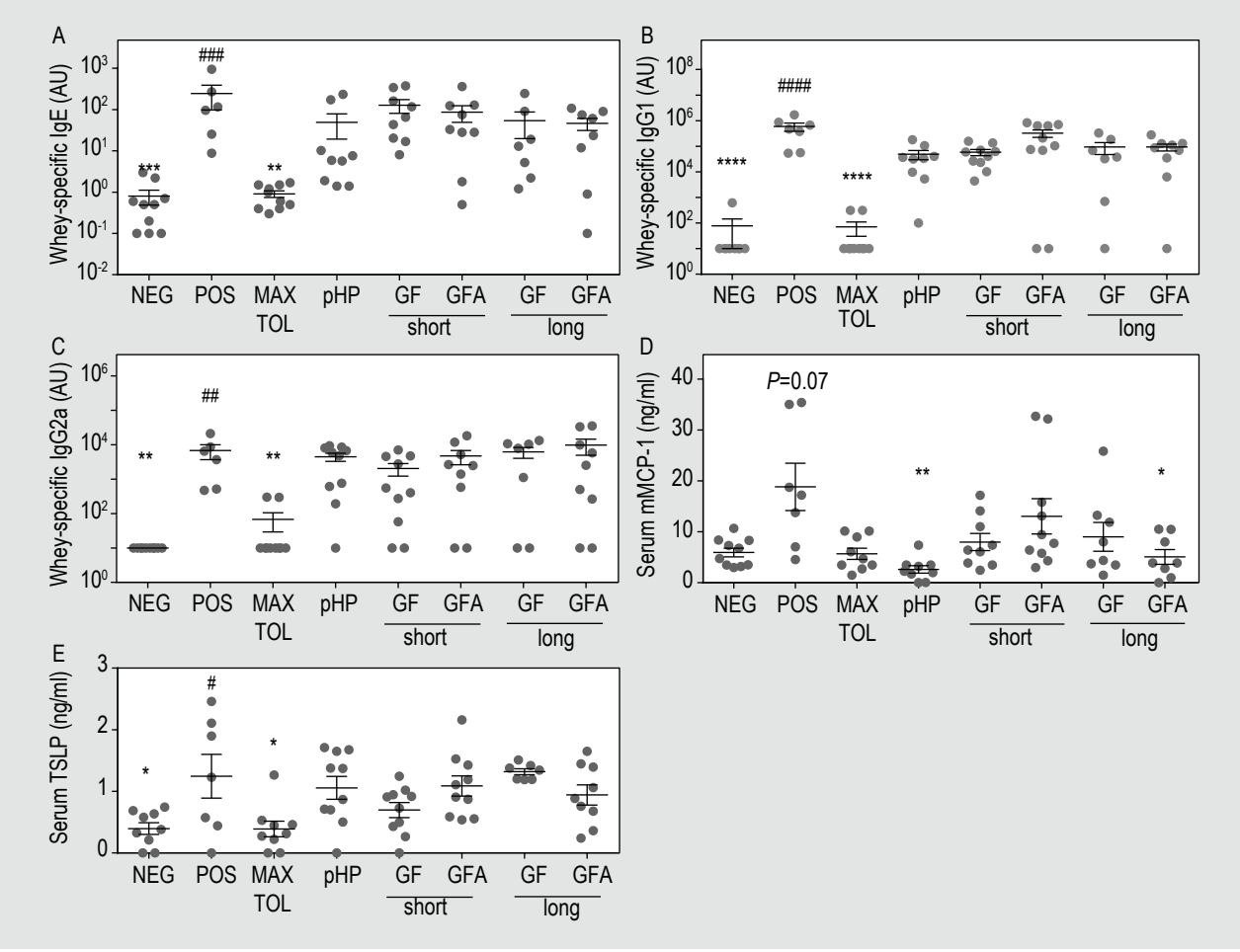

Figure 3. Allergen-induced serum marker concentrations 1 h after the oral whey-gavage. (A) Whey-specific immunoglobulin (Ig) E, (B) whey-IgG1, (C) whey-IgG2a, (D) mouse mast cell protease-1, and (E) thymic stromal lymphopoietin. Data are expressed as individual values with the group-mean ± standard error of the mean. Six outliers were removed from the IgE analysis (POS, n=6; pHP, n=9; GFshort, n=9, GFAshort, n=9; GFlong, n=7; GFAlong, n=8). Three outliers were removed from the IgG1 analysis (NEG, n=9; pHP, n=9; GFlong, n=7). Three outliers were removed from the IgG2a analysis (POS, n=6; GFAshort, n=9; GFlong, n=7). * *P*<0.05, ** *P*<0.01, * *P*<0.001, **** *P*<0.0001 compared to the POS group, and # *P*<0.05, ## *P*<0.01, ### *P*<0.001, #### *P*<0.0001 compared to the NEG group. For treatment abbreviations see Figure 1.**

4A) and the same trend was visible for all individual groups (Figure 4B). The median phylogenetic diversity at day -8 was higher in every group than the median at day -15. The change in phylogenetic diversity within the groups was only significant in the GFshort, GFAshort and GFlong groups. In the mice that did not become allergic, either due to the lack of sensitization (NEG group) or effective tolerance induction (MAXtol), the trend of increasing phylogenetic diversity continued until day 34, whereas in the POS animals and all pHP groups the diversity decreased compared to day -8. We hypothesise that in the GF(A)short groups, the effect of the prebiotic treatment already faded, whereas in the GF(A)long groups, the microbiota already adjusted to the continuous availability of prebiotics in the gut.

Microbial composition and dynamics

A principal component analysis (PCA) is an analysis method that can summarise multivariate data, e.g. microbial composition, into a smaller set of variables. Samples with a similar composition will be plotted closely together and plotted arrows represent the bacterial groups most responsible for the position of the samples. The three taxonomic groups that explain most of the observed microbiota variation are *Lactobacillus*, the *Bacteroidales* family *Bacteroidales* S24-7, and *Allobaculum* (Figure 5A). These present three of the four most abundant taxonomic groups with an average relative abundance of 27.0, 19.1 and 9.1% respectively. The high amount of microbiota variation explained by these groups is also due to the great inter-individual differences with minimum and maximum relative abundances of respectively 1.6-92.3%, 0.2-42.9%

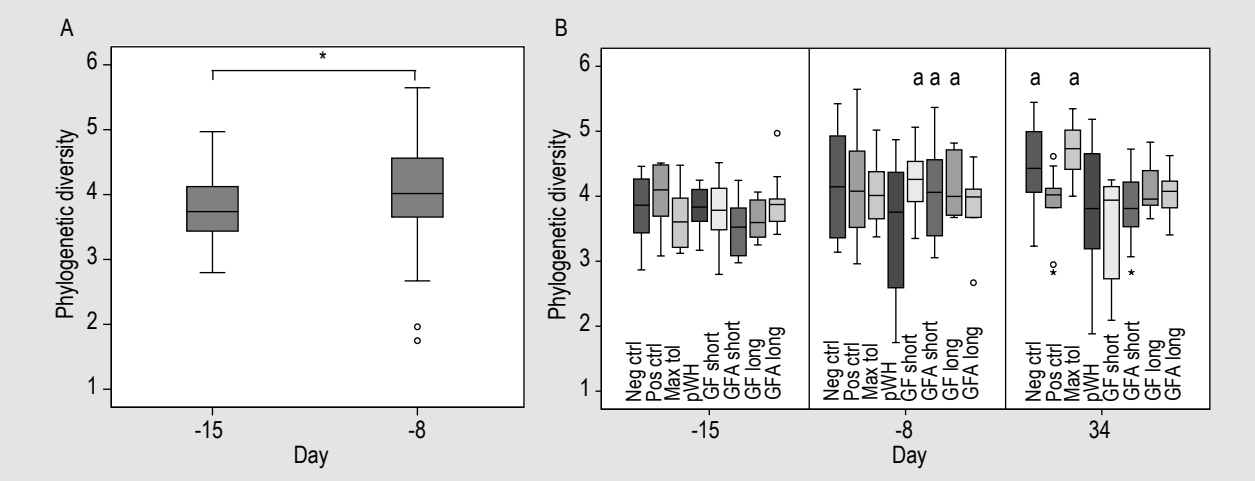

Figure 4. (A) Average phylogenetic diversity assessed within the faecal samples obtained before (day -15) and after (day -8) a run-in period. * Significant difference (*P*<0.05, paired t-test). (B) Group-specific phylogenetic diversity of the gut microbiota assessed over time. Samples marked with 'a' are significantly different from day -15 (*P*<0.05, paired t-test). ○ represent an outlier. For treatment abbreviations see Figure 1.

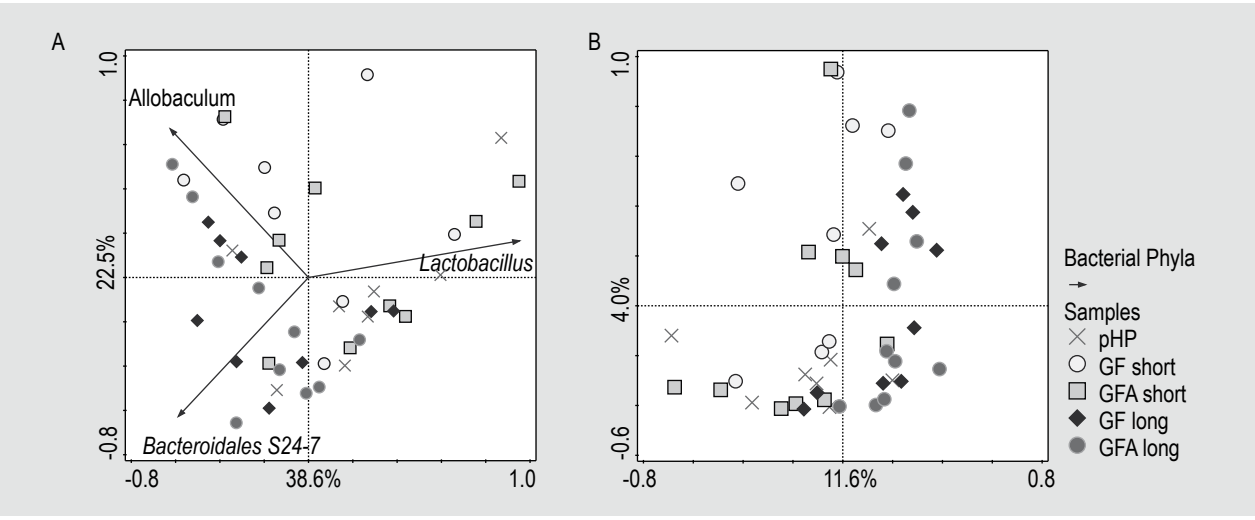

Figure 5. Multivariate analyses of microbial composition. (A) Principal component analysis. (B) Redundancy analysis with 'treatment' used as an explanatory variable and plotted as the x-axis. For treatment abbreviations see Figure 1.

and 0-49.3%. In the PCA, the samples of the GF(A)long groups are predominantly seen on the left side of the plot, as the samples of these treatments are associated mainly with *Allobaculum* and *Bacteroidales* S24-7 rather than with *Lactobacillus*. A redundancy analysis (RDA) is comparable to a PCA, but can also identify variables that explain variation in composition. These variables can be plotted as an axis to visualise its effect on microbiota coxfmposition. When 'treatment' was used as an explanatory variable in an RDA analysis (Figure 5B), it became apparent that it explains 11.6% of the observed variation. When 'treatment' was plotted as the first axis, an even clearer separation of the treatment groups was visible as compared to the PCA.

Principle response curve with significantly contributing strains

A principal response curve (PRC) is a special type of RDA; it is a multivariate statistical analysis method that allows for the study of time-dependent treatment effects (Van den Brink and Ter Braak, 1999). PRC plots can visualise how treatment groups differ from the control over time and which (microbial) variables are responsible for these differences. In this scenario, it enables us to identify bacterial taxa that have a different interaction with time in our prebiotic treatment groups as opposed to our reference (pHP) group. The plot shows the dynamics of the microbial taxa within the prebiotics groups as opposed to the baseline pHP group (Figure 6A). Strikingly, the groups with a

significantly reduced ear swelling response demonstrated the largest deviation from the pHP group. As indicated by the largest absolute value, the presence of *Lactobacillus* within the microbiota of the prebiotic groups deviated most from the pHP group over time. This effect was strongest in GFlong, subsequently followed by GFAlong, GFshort and GFAshort. At day -15, the microbiota was very similar in all groups. Towards day -8, there was a reduction in *Lactobacillus* and an increase in the other taxa displayed in Figure 6A. This difference was only expected in the GF(A)long groups, as they were already receiving prebiotics, in contrast to all other groups. The dynamics observed in the 'long' treatment groups were similar to those in the 'short' groups, only stronger. This indicates that prebiotics probably enhance the microbiota changes that are already taking place. From day -8 towards day 34, the microbial composition of the GFlong, GFshort and GFAshort became more similar to pHP. This return towards the reference group could be explained by the fact that the intervention with prebiotics was only given between day -8 and 0. Especially the GFAshort group remained close to the composition observed in the pHP group over time.

The distribution of the abundance of *Lactobacillus* at day 34 was significantly different in GFAlong group compared to the pHP group (Figure 6A). Although the GFlong group showed a comparable distribution to GFAlong, its difference in distribution with pHP was not statistically significant. The *Bacteroidales* family S24-7 was one of the most abundant taxa in our study. Although there are no significant differences in the abundance of the S24-7 family between pHP and any of the prebiotics groups, it was the

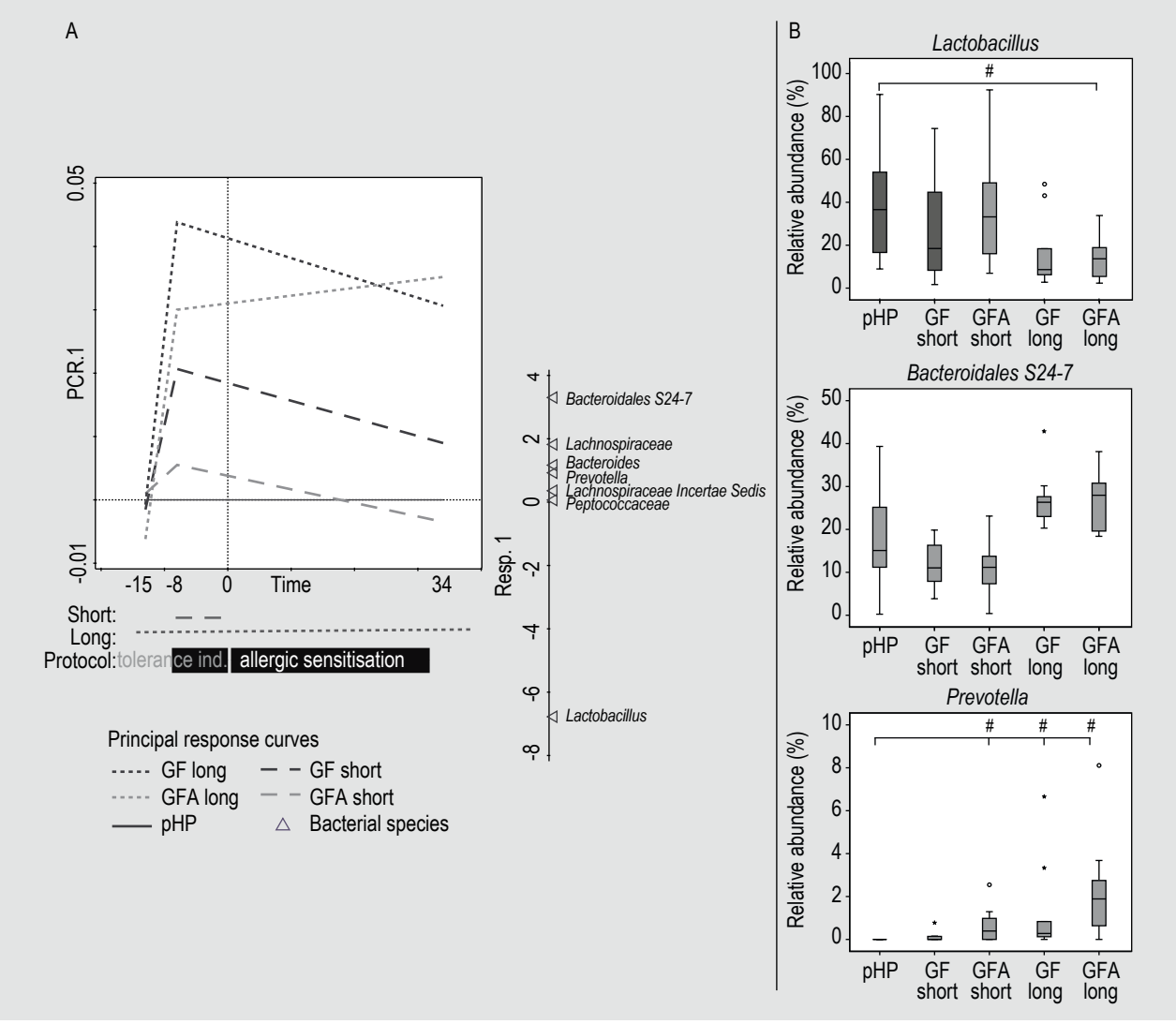

Figure 6. Principle response curves with significantly contributing strains. (A) Dynamics of the taxa within the prebiotics groups as opposed to the baseline pHP group. (B) The three taxa that are most affected in the PRC depicted to show the differences in abundance across treatment groups. # Indicate significant differences between groups. The ○ represent outliers and the small * extreme outliers. For treatment abbreviations see Figure 1.

group with second highest response value in the PRC after the *Lactobacillus* (Figure 5B). *Lactobacillus* was especially reduced in the GF(A) groups, whereas the *Bacteroidales* family S24-7 gained ground, however, this difference was not statistically significant. The relative abundance of taxon *Prevotella* (Figure 6B) was significantly increased in the GFAshort, GFlong and GFAlong groups when compared to pHP, which is partially due to its absence in the pHP group.

4. Discussion and conclusions

To test whether combining hydrolysed whey protein with prebiotics leads to better tolerance induction and to understand whether the gut microbiota plays a role in immunological tolerance induction, we co-administered mice with pHP and GF(A) in two different exposure durations. When pHP and prebiotics were administered during tolerance induction, GF but not GFA reduced the allergen-induced ear swelling and shock score. In contrast, both the administration of GF as well as GFA during tolerance induction and the sensitization phase significantly reduced the ear swelling.

The tolerance inducing capacity of different hydrolysates was studied before in multiple murine studies using the cow's milk allergy model. Van Esch *et al.* (2011b) used the cow's milk allergy model to show that oral tolerance induction with both whole protein as well as pHP led to a reduced immediate type hypersensitivity response. In contrast, this response was not achieved with extensively hydrolysed whey proteins. Prolonged exposure to specific oligosaccharides during sensitization has also been shown to elicit protective effects against the development of or the reduction of allergic symptoms in a murine cow's milk allergy model (Schouten *et al.*, 2010).

The direct effect of the used prebiotics was studied before by, amongst others, Kerperien *et al.*, (2014) who compared different compositions of prebiotic interventions. Besides single oligosaccharides, GF (9:1) and GFA (9:1:2) were tested in a model of cow's milk allergy. They showed that different oligosaccharide mixtures have a differential effect on mucosal immune activation; GF seemed to reduce the Th2-prone immune activation, whereas GFA appeared to enhance Th1 and Treg responses. Recently, Van Esch *et al.* (2017) showed that that a combination of non-digestible oligosaccharides and a partial whey hydrolysate prevents the onset of allergic symptoms and that dietary non-digestible oligosaccharides may optimise immune modulation and support oral tolerance induction by (hydrolysed) food proteins (Van Esch *et al.*, 2017).

Besides the immunological research, we investigated how the gut microbiome changed due to the immunological tolerance induction procedure with two different non-digestible oligosaccharide mixtures. By using a faecal sampling point before and after a run-in period, we showed that the microbiota composition of mice can change significantly after the mice arrive in a new facility. This observation stresses the importance of a run-in period, because it is important that the microbiota of all animals reaches a stable state before the start of an experiment in which the gut microbiota is expected to play an important role. As reported earlier, a high degree of facility-level individuality can be observed when mice are exposed to varying conditions at each facility, such as feed type, animal handling, cage type and bedding (Rausch *et al.*, 2016).

Also after the run-in period, the average phylogenetic diversity significantly increased over the course of the experiment. This was observed in some of the individual treatment groups from day -15 to -8 but the diversity did not increase in the GFAlong group. The absence of an increased diversity in GFAlong might be caused by the prebiotic treatment that the animals in this group were already undergoing, although the effect is absent in animals of the GFlong group, which were also already receiving prebiotics. Previous prebiotic intervention studies have shown increased microbiota diversity in animal studies (Konstantinov *et al.*, 2004a,b,c), whereas in infants the consumption of natural prebiotics in the form of human milk oligosaccharides (Marcobal *et al.*, 2010) has been associated with a decreased microbial diversity (Azad *et al.*, 2013; Bezirtzoglou *et al.*, 2011). Phylogenetic diversity continued to increase in the control mice that did not become allergic, due to either lack of sensitization (NEG) or effective tolerance induction (MAXtol). However, diversity decreased in mice that did not undergo tolerance induction and thus became allergic (POS, all pHP groups). Literature provides many examples of decreased diversity being linked to allergy (Abrahamsson *et al.*, 2014; Nylund *et al.*, 2015). In these studies, it is suggested that allergy is being caused by a low diversity, however, our data suggest that maybe the activated immune system halts the increase in phylogenetic diversity in the allergic mice. Maybe the mice that became allergic not only developed a hypersensitivity against cow's milk protein, but also against newly acquired members of the gut microbiota.

Functional study of the microbiota could provide better insights into the mechanisms by which it affects the onset of allergy. Higher levels of the bioactive short-chain fatty acid (SCFA) butyrate have been found before in infants suffering from cow's milk protein allergy (Thompson-Chagoyan *et al.*, 2011). We did collect the caecal contents of the mice and analyse SCFA concentrations, but did not find any significant differences between the treatment groups.

The three taxonomic groups that explain most of the observed microbiota variation in general are *Lactobacillus*, the family *Bacteroidales* S24-7, and *Allobaculum*. *Lactobacillus* is commonly found in the mouse gut

microbiota, in much higher relative abundances than in humans, often as the most common genus (Nguyen *et al.*, 2015). *Bacteroidales* S24-7 is also commonly found in the mouse gut microbiota, it was actually first recognised in a 16S rRNA gene clone library survey of the mouse microbiota (Salzman *et al.*, 2002). *Allobaculum* is also frequently found in the gut microbiota of mice, often negatively correlated with high fat feeding, leptin levels and fat mass (Cox *et al.*, 2014; Everard *et al.*, 2011; Ravussin *et al.*, 2012).

None of the bacterial taxa consistently correlated with all the treatment groups that had a reduced allergic response. Nonetheless, we found that treatment still explained 11.6% of the observed variation in an RDA. The largest effect on microbiota composition was expected in the GFlong and GFAlong groups, since the animals in these treatment groups had been receiving a diet with prebiotics up until the sampling point. Variance explained by treatment could also be interpreted as an activation of the immune system in allergic mice, favouring certain members of the gut microbiota over others.

The distribution of the abundance of *Lactobacillus* at day 34 is significantly different in GFAlong as compared to GFlong. GFlong has a comparable distribution, but this difference is not significant because of outliers. In human intervention studies, however, an increase of *Lactobacillus* has been observed (Bryk *et al.*, 2015; Haarman and Knol, 2006; Mika *et al.*, 2014). The difference in average relative abundance of *Lactobacillus* between mice and humans could explain why an increase in *Lactobacillus* is not so obvious here. Possibly, there are groups that are more efficient degraders of the prebiotic fibres in the mouse microbiota, such as members of the *Bacteroidales* order, e.g. the *Bacteroidales* family S24-7 or *Prevotella*. The *Bacteroidales* family S24-7 is one of the most abundant taxa in our study, reaching the highest levels in the 'long' treatment groups. This higher relative abundance could indicate that members of this family are feeding of the prebiotics. A recent study identified three trophic guilds within the family, specialised in either α-glucan, host glycan or plant glycan-based carbohydrates (Ormerod *et al.*, 2016). The second and third aforementioned groups might be responsible for the increase seen in our 'long' treatment groups. Such an increase is similar to the increase in S24-7 observed after supplementation with GOS alone (Serino *et al.*, 2012).

The decrease over time in *Lactobacillus* is accompanied by an increase of *Bacteroidales* S24-7 in the prebiotic treatment groups as compared to the pHP control. The deviation from pHP was strongest in the GFlong and GFAlong groups followed by GFshort group. The effect was the smallest in GFAshort, which is interesting since the animals in this group had no significantly reduced ear swelling and shock score. We are not the first to report a trade-off between *Lactobacillus* and *Bacteroidetes*; this was also seen in a study where mice were treated with vancomycin, which made them more susceptible to allergic asthma (Russell *et al.*, 2012). The authors propose that the depletion of *Bacteroides* species could have increased the susceptibility of the mice to allergic asthma since some strains have been implicated in Treg differentiation (Atarashi *et al.*, 2011; Round and Mazmanian, 2010). Additional evidence for the association of *Bacteroides* with asthma is provided by the observation that its levels are reduced in C-section born children, which in turn is negatively correlated with an increased risk of developing childhood asthma (Azad *et al.*, 2013; Murk *et al.*, 2011; Van Nimwegen *et al.*, 2011).

Prevotella's relative abundance is significantly increased in the GFAshort, GFlong and GFAlong groups when compared to pHP, which is partially due to its absence in the pHP group. This genus, known for its bimodal distribution in humans (Lahti *et al.*, 2014) and for being characteristic of one of the three enterotypes (Arumugam *et al.*, 2011), is known to have a low abundance in mice (Hildebrand *et al.*, 2013), 0.9% on average, based on 5 studies (Nguyen *et al.*, 2015). High abundance of *Prevotella* and the *Prevotella* enterotype are associated with a diet high in sugars and fibres in humans (De Filippo *et al.*, 2010; Wu *et al.*, 2011), indicating a preference of *Prevotella* for carbohydrates. In a study using wheat arabinoxylan as a prebiotic for mice, *Prevotella* abundance was increased and negatively correlated with weight gain, adiposity, cholesterol accumulation and insulin resistance (Neyrinck *et al.*, 2011). The preference of *Prevotella* for carbohydrates most likely explains why it is present in the prebiotic groups and absent in the pHP controls.

In conclusion, the current study demonstrates that combining pHP with prebiotic mixtures improves allergic tolerance induction as compared to tolerance induction with pHP alone. Our data suggest an interplay between immunological and microbiological mechanisms which support oral tolerance induction by (hydrolysed) food proteins.

References

Abrahamsson, T.R., Jakobsson, H.E., Andersson, A.F., Bjorksten, B., Engstrand, L. and Jenmalm, M.C., 2014. Low gut microbiota diversity in early infancy precedes asthma at school age. Clinical and Experimental Allergy 44: 842-850.

Arslanoglu, S., Moro, G. E., Schmitt, J., Tandoi, L., Rizzardi, S. and Boehm, G., 2008. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. Journal of Nutrition 138: 1091-1095.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H.B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E.G. Wang, J., Guarner, F., Pedersen, O., de Vos, W.M., Brunak, S., Dore, J., Meta, H.I.T.C., Antolin, M., Artiguenave, F., Blottiere, H.M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K.U., Friss, C., Van de Guchte, M., Guedon, E., Haimet, F., Huber, W., Van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Merieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S.D. and Bork, P., 2011. Enterotypes of the human gut microbiome. Nature 473: 174-180.

Atarashi, K., Umesaki, Y. and Honda, K., 2011. Microbiotal influence on T cell subset development. Seminars in Immunology 23: 146-153.

Azad, M.B., Becker, A.B., Guttman, D.S., Sears, M.R., Scott, J.A., Kozyrskyj, A.L. and Canadian Healthy Infant Longitudinal Development Study, 2013. Gut microbiota diversity and atopic disease: does breast-feeding play a role? Journal of Allergy and Clinical Immunology 131: 247-248.

Bezirtzoglou, E., Tsiotsias, A. and Welling, G.W., 2011. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence *in situ* hybridization (FISH). Anaerobe 17: 478-482.

Boyle, R.J., Tang, M.L., Chiang, W.C., Chua, M.C., Ismail, I., Nauta, A., Hourihane, J.O., Smith, P., Gold, M., Ziegler, J., Peake, J., Quinn, P., Rao, R., Brown, N., Rijnierse, A., Garssen, J., Warner, J.O. and PATCH Study Investigators, 2016. Prebiotic-supplemented partially hydrolysed cow's milk formula for the prevention of eczema in high-risk infants: a randomized controlled trial. Allergy 71: 701-710.

Bryk, G., Coronel, M.Z., Pellegrini, G., Mandalunis, P., Rio, M.E., De Portela, M.L. and Zeni, S.N., 2015. Effect of a combination GOS/FOS(R) prebiotic mixture and interaction with calcium intake on mineral absorption and bone parameters in growing rats. European Journal of Nutrition 54: 913-923.

Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y. and Powrie, F., 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. Journal of Experimental Medicine 204: 1757-1764.

Cox, L.M., Yamanishi, S., Sohn, J., Alekseyenko, A.V., Leung, J.M., Cho, I., Kim, S.G., Li, H., Gao, Z. and Mahana, D., 2014. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell 158: 705-721.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G. and Lionetti, P., 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Science of the USA 107: 14691-14696.

Everard, A., Lazarevic, V., Derrien, M., Girard, M., Muccioli, G.G., Neyrinck, A.M., Possemiers, S., Van Holle, A., Francois, P., De Vos, W.M., Delzenne, N.M., Schrenzel, J. and Cani, P.D., 2011. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60: 2775-2786.

Fujimura, K.E. and Lynch, S.V., 2015. Microbiota in allergy and asthma and the emerging relationship with the gut microbiome. Cell Host and Microbe 17: 592-602.

Haarman, M. and Knol, J., 2006. Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. Applied and Environmental Microbiology 72: 2359-2365.

Hildebrand, F., Nguyen, T.L., Brinkman, B., Yunta, R.G., Cauwe, B., Vandenabeele, P., Liston, A. and Raes, J., 2013. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biology 14: R4.

Iwasaki, A., 2007. Mucosal dendritic cells. Annual Review of Immunology 25: 381-418.

Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J.L., Berg, P.L., Davidsson, T., Powrie, F., Johansson-Lindbom, B. and Agace, W.W., 2008. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. Journal of Experimental Medicine 205: 2139-2149.

Kerperien, J., Jeurink, P.V., Wehkamp, T., Van der Veer, A., Van de Kant, H.J., Hofman, G.A., Van Esch, E.C., Garssen, J., Willemsen, L.E. and Knippels, L.M., 2014. Non-digestible oligosaccharides modulate intestinal immune activation and suppress cow's milk allergic symptoms. Pediatric Allergy and Immunology 25: 747-754.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41: e1.

Konstantinov, S.R., Awati, A., Smidt, H., Williams, B.A., Akkermans, A.D. and De Vos, W.M., 2004a. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. Applied and Environmental Microbiology 70: 3821-3830.

Konstantinov, S.R., Awati, A., Smidt, H., Williams, B.A., Akkermans, A.D. and De Vos, W.M., 2004b. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. Applied and Environmental Microbiology 70: 3821-3830.

Konstantinov, S.R., Favier, C.F., Zhu, W.Y., Williams, B.A., Klüβ, J., Souffrant, W.-B., De Vos, W.M., Akkermans, A.D. and Smidt, H., 2004c. Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. Animal Research 53: 317-324.

Lahti, L., Salojarvi, J., Salonen, A., Scheffer, M. and De Vos, W.M., 2014. Tipping elements in the human intestinal ecosystem. Nature Communications 5: 4344.

Lynch, S.V., 2016. Gut microbiota and allergic disease. New insights. Annals of the American Thoracic Society 13 (Suppl. 1): S51-S54.

Marcobal, A., Barboza, M., Froehlich, J.W., Block, D.E., German, J.B., Lebrilla, C.B. and Mills, D.A., 2010. Consumption of human milk oligosaccharides by gut-related microbes. Journal of Agricultural and Food Chemistry 58: 5334-5340.

Mika, A., Greenwood, B., Chichlowski, M., Borchert, D., Hulen, K., Berg, B., Paton, M. and Fleshner, M., 2014. 155. Dietary prebiotics increase *Bifidobacterium* spp. and *Lactobacillus* spp. in the gut and promote stress resistance. Brain, Behavior, and Immunity 40: e45.

Moro, G., Arslanoglu, S., Stahl, B., Jelinek, J., Wahn, U. and Boehm, G., 2006. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Archives of Disease in Childhood 91: 814-819.

Murk, W., Risnes, K.R. and Bracken, M.B., 2011. Prenatal or early-life exposure to antibiotics and risk of childhood asthma: a systematic review. Pediatrics 127: 1125-1138.

Neyrinck, A.M., Possemiers, S., Druart, C., Van de Wiele, T., De Backer, F., Cani, P.D., Larondelle, Y. and Delzenne, N.M., 2011. Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, *Roseburia* and *Bacteroides*/*Prevotella* in diet-induced obese mice. PLoS ONE 6: e20944.

Nguyen, T.L., Vieira-Silva, S., Liston, A. and Raes, J., 2015. How informative is the mouse for human gut microbiota research? Disease Models & Mechanisms 8: 1-16.

Nylund, L., Nermes, M., Isolauri, E., Salminen, S., De Vos, W.M. and Satokari, R., 2015. Severity of atopic disease inversely correlates with intestinal microbiota diversity and butyrate-producing bacteria. Allergy 70: 241-244.

Ormerod, K.L., Wood, D.L., Lachner, N., Gellatly, S.L., Daly, J.N., Parsons, J.D., Dal'Molin, C.G., Palfreyman, R.W., Nielsen, L.K., Cooper, M.A., Morrison, M., Hansbro, P.M. and Hugenholtz, P., 2016. Genomic characterization of the uncultured *Bacteroidales* family S24-7 inhabiting the guts of homeothermic animals. Microbiome 4: 36.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41: D590-D596.

Ramiro-Garcia, J., Hermes, G.D., Giatsis, C., Sipkema, D., Zoetendal, E.G. Schaap, P.J. and Smidt, H., 2016. NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000 Research 5: 1791.

Rausch, P., Basic, M., Batra, A., Bischoff, S.C., Blaut, M., Clavel, T., Glasner, J., Gopalakrishnan, S., Grassl, G.A., Gunther, C., Haller, D., Hirose, M., Ibrahim, S., Loh, G., Mattner, J., Nagel, S., Pabst, O., Schmidt, F., Siegmund, B., Strowig, T., Volynets, V., Wirtz, S., Zeissig, S., Zeissig, Y., Bleich, A. and Baines, J.F., 2016. Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. International Journal of Medical Microbiology 306: 343-355.

Ravussin, Y., Koren, O., Spor, A., LeDuc, C., Gutman, R., Stombaugh, J., Knight, R., Ley, R.E. and Leibel, R.L., 2012. Responses of gut microbiota to diet composition and weight loss in lean and obese mice. Obesity 20: 738-747.

Round, J.L. and Mazmanian, S.K., 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences of the USA 107: 12204-12209.

Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.R., Mohn, W.W., McNagny, K.M. and Finlay, B.B., 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Reports 13: 440-447.

Salzman, N.H., De Jong, H., Paterson, Y., Harmsen, H.J., Welling, G.W. and Bos, N.A., 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology 148: 3651-3660.

Schouten, B., Van Esch, B.C., Hofman, G.A., Boon, L., Knippels, L.M., Willemsen, L.E. and Garssen, J., 2010. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. Journal of Nutrition 140: 835-841.

Schouten, B., Van Esch, B.C., Hofman, G.A., De Kivit, S., Boon, L., Knippels, L.M., Garssen, J. and Willemsen, L.E., 2012. A potential role for CD25+ regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. British Journal of Nutrition 107: 96-105.

Serino, M., Luche, E., Gres, S., Baylac, A., Berge, M., Cenac, C., Waget, A., Klopp, P., Iacovoni, J., Klopp, C., Mariette, J., Bouchez, O., Lluch, J., Ouarne, F., Monsan, P., Valet, P., Roques, C., Amar, J., Bouloumie, A., Theodorou, V. and Burcelin, R., 2012. Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. Gut 61: 543-553.

Ter Braak, C.J., Cormont, A. and Dray, S., 2012. Improved testing of species traits – environment relationships in the fourth-corner problem. Ecology 93: 1525-1526.

Thompson-Chagoyan, O. C., Fallani, M., Maldonado, J., Vieites, J. M., Khanna, S., Edwards, C., Doré, J. and Gil, A., 2011. Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy. International Archives of Allergy and Immunology 156: 325-332.

Tian, Q., Zhao, W., Lu, S., Zhu, S. and Li, S., 2016. DNA barcoding for efficient species- and pathovar-level identification of the quarantine plant pathogen *Xanthomonas*. PLoS ONE 11: e0165995.

Van den Brink, P.J. and Ter Braak, C.J., 1999. Principal response curves: analysis of time-dependent multivariate responses of biological community to stress. Environmental Toxicology and Chemistry 18: 138-148.

Van Esch, B.C., Knipping, K., Jeurink, P., Van der Heide, S., Dubois, A.E., Willemsen, L.E., Garssen, J. and Knippels, L.M., 2011a. *In vivo* and *in vitro* evaluation of the residual allergenicity of partially hydrolysed infant formulas. Toxicology Letters 201: 264-269.

Van Esch, B.C., Kostadinova, A., Garssen, J., Willemsen, L.E. and Knippels, L.M., 2017. A dietary intervention with non-digestible oligosaccharides and partial hydrolysed whey protein prevents the onset of food allergic symptoms in mice. PharmaNutrition 5: 1-7.

Van Esch, B.C., Schouten, B., De Kivit, S., Hofman, G.A., Knippels, L.M., Willemsen, L.E. and Garssen, J., 2011b. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+ regulatory T-cells in the mesenteric lymph nodes. Pediatric Allergy and Immunology 22: 820-826.

Van Nimwegen, F.A., Penders, J., Stobberingh, E.E., Postma, D.S., Koppelman, G.H., Kerkhof, M., Reijmerink, N.E., Dompeling, E., Van den Brandt, P.A., Ferreira, I., Mommers, M. and Thijs, C., 2011. Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. Journal of Allergy and Clinical Immunology 128: 948-955.

Wopereis, H., Oozeer, R., Knipping, K., Belzer, C. and Knol, J., 2014. The first thousand days – intestinal microbiology of early life: establishing a symbiosis. Pediatric Allergy and Immunology 25: 428-438.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F.D. and Lewis, J.D., 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science 334: 105-108.

Ziegler, S.F., 2010. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. Current Opinion in Immunology 22: 795-799.