doi: 10.1111/cea.12905
Clinical & Experimental Allergy, **47**, 805–814

ORIGINAL ARTICLE **Experimental Models of Allergic Disease**

The farming environment protects mice from allergen-induced skin contact hypersensitivity

C. P. Frossard[1], V. Lazarevic[2,3], N. Gaïa[2,3], S. Leo[2,3], C. Doras[4], W. Habre[4], J. Schrenzel[2,3], D. Burger[1,5] and P. A. Eigenmann[1]

[1]Inflammation and Allergy Research Group, University Hospitals of Geneva and University of Geneva, Geneva, Switzerland, [2]Genomic Research Laboratory, Division of Infectious Diseases, Departments of Pediatrics, Anesthesiology, and Internal Medicine Specialties, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [3]Division of Laboratory Medicine, Departments of Pediatrics, Anesthesiology, and Internal Medicine Specialties, Faculty of Medicine, University of Geneva, Geneva, Switzerland, [4]Division of Anesthesiology Investigation, Departments of Pediatrics, Anesthesiology, and Internal Medicine Specialties, Faculty of Medicine, University of Geneva, Geneva, Switzerland and [5]Hans Wilsdorf Laboratory, Departments of Pediatrics, Anesthesiology, and Internal Medicine Specialties, Faculty of Medicine, University of Geneva, Geneva, Switzerland

Clinical & Experimental Allergy

Correspondence:
Philippe A Eigenmann, Pediatric Allergy Unit, University Hospitals of Geneva, 6 rue Willy-Donze, 1211 Geneva 14, Switzerland.
E-mail: Philippe.Eigenmann@hcuge.ch
Cite this as: C. P. Frossard, V. Lazarevic, N. Gaïa, S. Leo, C. Doras, W. Habre, J. Schrenzel, D. Burger and P. A. Eigenmann, *Clinical & Experimental Allergy*, 2017 (47) 805–814.

Summary

Background Being born and raised in a farm provides a long-lasting protection for allergies. The microbial environment provided by farm animals is crucial to induce this protective effect, although underlying immune mechanisms remain elusive.

Objective To establish a mouse model of global exposure to the farming environment and to study immunologic changes linked to protection of allergy.

Methods Mice colonies were bred in parallel in a farm cowshed and the university animal facility (AF). Mice from both locations were subjected to a skin contact allergy model. Peripheral blood cells and cell cytokine production were assessed in both populations. In addition, the gut microbiome at various ages was characterized.

Results Mice born in the farm were less prone to develop allergy than mice bred in the AF. Mice transfers between the AF and the farm showed a better protection when mice were moved to the farm early in life. As compared to AF-bred mice, farm mice displayed early immune activation with higher $CD4^+$ T cell population, in particular $CD4^+CD25^+FoxP3^-$ (activated cells). The cytokine profile of mice from the farm was skewed towards an IL-17 and IL-22 secreting cell profile accompanied by increased IL-10 secretion. These differences were mostly seen within a specific age window between birth and 8 weeks of age. Microbiome analysis showed differences between 4 and 20 weeks old mice and between farm and AF mice with an increased number of Murine mastadenovirus B in young farm mice exclusively.

Conclusion The farming environment provides a strong, allergy protective IL-22 stimulus and generates activated CD4+ T cells. Exposure to the farm environment early in their life may also provide a better protection for contact skin allergy. Whether a viral trigger might decisively influence protection for allergies remains to be determined.

Keywords allergy, farming environment, gut microbiome, Mastadenoviruses, T cell tolerance
Submitted 17 October 2016; revised 6 January 2017; accepted 8 February 2017

Introduction

The pathogenesis of allergy is multi-factorial and mostly influenced by early life factors, such as the environment and genetic factors [1]. In the environment, various micro-organisms have been identified to be protective for allergy [2, 3]. Among specific environmental exposures, the farm, loaded with microbial compounds due to the presence of cattle and other animals, has been recognized as a strongly protective environment for atopy in young children. The initial observations were made in a rural Alpine area of Switzerland, where children raised in farms were found to have less allergies than children living in the same area, but not in a farm [4]. A cross-sectional study including farmers' and non-farmers' children from

Austria, Germany and Switzerland, confirmed that the 'protective farming effect' was more specifically observed in children regularly exposed to farm animals, and consuming non-pasteurized milk when younger than 1 year. These children had lower frequencies of asthma (1% vs. 11%), hay fever (3% vs. 13%) and atopic sensitization (12% vs. 29%), when compared to non-farmers' children [5]. Further studies thereafter confirmed the strong protective effect of the farming environment on allergy, mostly related to factors robustly stimulating the innate immune system [6]. Only a few specific factors provided by the farming environment, that is consumption of non-pasteurized milk, exposure to farm pigs, and exposure to hay were crucial for benefiting from the protective 'farming effect' [6]. Finally, contact with farm animals in early childhood reduces the risk of atopic sensitization in adulthood suggesting long-lasting protective effects [7]. These observations raise a common hypothesis, that is a strong activation of the innate immune system with microbial products may boost organisms to an immune status less prone to develop allergies. The importance of the innate system activation was substantiated by the role of Toll-like receptors (TLR), leading to markers of activation such as CD14, a cell surface protein found up-regulated in protected children [8]. Although biological mechanisms underlying protective effects are still elusive, a farming environment generates epigenetic changes that might be involved in allergy protection. For instance, cord blood cells from infants born in a farming environment were found to have increased DNA methylation of genes related to asthma (*ORMDL* family) and IgE regulation (*RAD50*, *IL13* and *IL4*) [9]. Regulatory mechanisms could also appear later in life, as suggested by the same study in which DNA methylation in T regulatory genes (*FOXP3*, *RUNX3*) was not observed in cord blood [9], while the number of T regulatory cells was increased in 4.5 years old farmers' children from the same cohort study [10].

To investigate preventive strategies derived from the 'farming effect', the role of two abundant bacteria found in cowshed, *Acinetobacter lwoffii* F78 (Gram-negative) and *Lactococcus lactis* G121 (Gram-positive), was explored in an experimental mouse model of antigen-specific asthma [11]. Isolates from both bacteria were able to reduce allergic reactions in mice, to activate mammalian cells *in vitro* and to induce a Th1-polarizing programme in dendritic cells. Nevertheless, this approach by selecting candidate micro-organisms might lack other specific protective microorganisms or the synergistic effect of the large number of microbial components of the gut microbiome.

As various factors present in the farming environment might synergistically be protective for allergies, we aimed to develop an experimental approach by exposing mice to a 'global farming environment', that is by establishing colonies of mice in a cattle barn to explore occurrence of allergy and to study the mechanisms of the 'protective farming effect'. Mice groups bred at either location, or moved later in life to the farm, were experimentally sensitized by antigens through the skin in order to measure potential changes in allergic response modulated by the farming environment as well as the age-related timing of exposure. In addition, we characterized the immune response and compared it to mice bred in the conventional environment of a university animal facility (AF).

Methods

Mice

BALB/c mice (Charles River, L'Arbesle, France), or when specified FoxP3-GFP knock-in mice from the Balb/C background [12], were bred into subsequent generations in the cattle barn of a typical Alpine farm in rural western Switzerland. The mice were located in the same area than the cows with dimed daylight, and thus naturally exposed to cows and hay dust. Experiments were conducted from late spring through autumn. Control mice were bred in the conventional AF of the University of Geneva, School of Medicine, under SPF conditions. Unless specified, F2 generation animals were used for all experiments (Fig. 1). Animal housing was following university guidelines, and all experiments were approved by the Experimental Ethics Committee of the

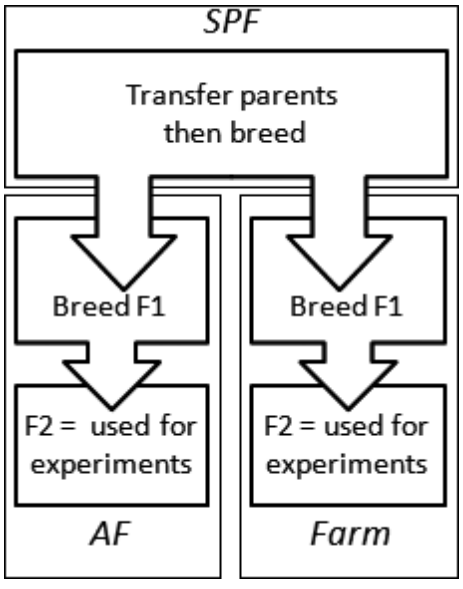


Fig. 1. Protocol for mice breeding. Mice from specific pathogen-free facilities (SPF) were split in two groups, one of them being transferred to the university animal facility (AF), the other to the farm. Experiments were carried out with mice from F2 generations.

University of Geneva and supervised by the Animal Welfare Committee of the Canton of Geneva, Switzerland.

Contact hypersensitivity model

Mice were primarily sensitized by painting 200 μL of 0.5% FITC in acetone/dibuthyl-phtalate (1/1) (all from Sigma, St. Louis, MO, USA) on the shaved abdomen. After 7 days, mice were challenged by application of 20 μL of 0.05% FITC in acetone/dibuthyl-phtalate (1/1) onto the mouse ears. The ear thickness was measured with a micrometer (Interapid, Rolle, Switzerland) 48 h after application of the antigen on one ear and the diluting solution without antigen on the other ear. The results were expressed as the difference between both measures.

Antibodies, reagents and media

Anti-CD3, anti-CD4, anti-CD8α, anti-CD19, anti-CD25, anti-CD45 and anti-NK were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). 7-amino-actinomycin D (7-AAD) was from Sigma. rmIL-2 and anti-CD3/CD28 beads were purchased from Life Technologies (Paisley, UK). The cell culture media RPMI 1640, DMEM and HBSS were supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 100 μg/mL gentamicin, 15 mM HEPES pH 7.4 and 10% heat-inactivated FCS. In addition, DMEM was supplemented 2×10^{-5} M 2-mercaptoethanol, 1% non-essential amino acids and 1 mM sodium pyruvate (all reagents from Sigma).

Measurement of antigen-specific antibodies in mouse serum

Sera were obtained by tail vein bleeding and stored at −20°C until analysis. FITC-specific Ab titres were measured by homemade ELISA. Briefly, Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg Ovalbumin (OVA) for 3 h at room temperature, followed by an incubation of 3 h with 0.1 μg of FITC (both from Sigma). The sera were serially diluted in ELISA buffer (PBS containing 10% horse serum) and incubated for 4 h at 37°C. Corresponding peroxidase-labelled monoclonal rat antibodies to mouse IgE (Pharmingen) or polyclonal goat antibodies to mouse IgG1, mouse IgG2a and mouse-IgG3 (Southern Biotechnologies, Birmingham, AL, USA) were added at 1/1000 in ELISA buffer for 90 min at 37°C. IgE was then detected with sheep anti-rat peroxidase-labelled Abs (AbD Serotec, Dusseldorf, Germany) at 1/1000 in ELISA buffet for 60 min at 37°C. After addition of ortho-phenylenediamine and H_2O_2 (Sigma), absorbance was measure at 490 nm on a plate reader (Molecular Device Corporation, Sunnyvale, CA, USA). Results were analyzed with the SoftMax™ software (Molecular Device Corporation) and normalized with a reference serum consisting in pooled sera from OVA and alum-immunized mice and expressed as arbitrary units.

Isolation of lymphocytes

Venous blood was collected in 250 U/mL Heparin (Roche, Reinach, Germany). Lymphocytes were enriched by Lympholyte M gradient centrifugation (Cederland, Hornby, Canada) as described by the manufacturer, for 20 min at 600 g at room temperature. Spleen cells were obtained after sacrifice of the mice by crushing the spleen, followed by red blood cells lysis. The cell suspension was incubated in complete RPMI, washed twice and loaded on discontinuous 15/30/44% Percoll (Bioscience, Uppsala, Sweden) upon a Lympholyte M gradient for 20 min at 600 g at room temperature. Lymphocytes were harvested from the Percoll 44% Lympholyte M interface.

Activated T cell cytokine profiles

Two $\times 10^5$ spleen cells per condition in complete DMEM were incubated in U bottom 96 wells plates with 50 U/mL of rmIL-2 or anti-CD3/CD28 beads (one bead per cell) for 3 days. Supernatants were harvested and stored at −20°C until analysis. Cytokines were quantified in the supernatants by Multiplex kits (Biolegend, San Diego, CA, USA) in accordance with manufacturer instructions. Due to the small sample amount, supernatants of five mice from the same litter were pooled; all experiments were repeated three times.

Flow cytometry

To block Fc receptors, cells were pre-incubated for 20 min on ice with purified rat IgG (Sigma) followed by incubation with labelled antibodies for 30 min on ice, then immediately analysed. Multi-parameter analysis was acquired on a flow cytometer (FACSCalibur™, BD Bioscience, Franklin Lakes, NJ, USA), and the data processed with the CellQuest™ software (BD Bioscience). To analyze lymphoid cells, we gated on CD45+ cells, and for analysis of T lymphocyte subpopulations, we gated on CD3+ cells. Dead and necrotic cells stained with 7-AAD were excluded from the analysis.

DNA extraction from faeces

A stool sample (about 100 mg) was mixed with 700 μL of GT buffer (RBC Bioscience, New Taipei City, Taiwan). The mixture was shaken in a NucleoSpin Bead Tube containing ceramic beads (Macherey-Nagel, Düren, Germany) for 20 min at maximum speed on a Vortex-Genie 2 with a horizontal tube holder (Scientific

Industries, Bohemia, NY, USA) and then centrifuged for 1 min at 11 000 g. DNA was extracted from 400 μL of the supernatant using the MagCore Genomic DNA Tissue Kit on a MagCore HF16 automated nucleic acid extractor (RBC Bioscience). DNA concentration was measured using a Qubit dsDNA BR Assay Kit on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Purified DNA was stored at -20°C.

Sequencing

Metagenomic libraries were prepared from 1 ng DNA using Nextera XT DNA Sample Preparation Kit according to Illumina (San Diego, CA, USA) instructions. The pooled indexed library was sequenced at Fasteris (Planles-Ouates, Switzerland) in Rapid Run mode from both ends for 250 cycles on the Illumina HiSeq 2500 using HiSeq Rapid SBS Kit. The trimmomatic package [13] was used to remove bases that correspond to the standard Illumina adapters.

Taxonomic analysis

Paired reads were joined using PEAR [14] with the following parameters –m 470 –n 200 –v 30 –q 33 –t 150 –p 0.0001 –u 0. Reads without matched pairs were removed. Merged FASTQ files for each sample were deposited in MG-RAST (project mgp21090). To filter out putative artificial replicate reads, we used a homemade script which retains only forward reads with the longest sequence among those with identical first 100 bases. Merged metagenomic reads were classified at the genus level against complete bacterial, archaeal and viral genome NCBI/RefSeq database) with Clark [15] in full mode, using discriminative 31-mers. Shannon diversity index was calculated from the relative abundance of genera in PRIMER (Primer-E Ltd., Plymouth, UK) after rarefying the number of classified reads to 29 813 (the lowest number of classified sequences in any sample).

Statistical analysis and community clustering

Immunologic data were expressed as mean ± SEM. Statistical significance between groups was analyzed by using the Wilcoxon signed-rank test for non-parametric paired, and the Wilcoxon rank-sum test for unpaired data. Bacterial community comparisons were carried out using Bray–Curtis [16] similarity matrix, based on the relative abundance of bacterial genera. Principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) of the Bray–-Curtis similarity matrix were performed in PRIMER (Primer-E Ltd., Plymouth, UK). Statistical significance was set at the 95% confidence level ($P < 0.05$).

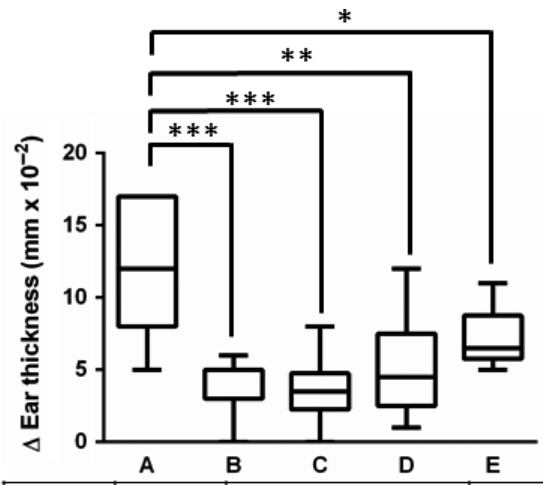
Results

Clinical status of mice bred in the farm and in the AF

All mice were originally purchased from an SPF animal facility. Breeding was then performed at the barn of the farm and the AF. All experiments were carried out with F2 generation mice (see Fig. 1). Mice were visually monitored for good health, and their weight was measured on a monthly basis. Overall mice were in good health at both places, with an identical weight for both groups, irrespective of gender. As the only notable difference, in the farm, offsprings were born only between spring and fall, whereas birth occurred throughout the year at the AF.

Farming environment protects mice for allergen-induced skin contact hypersensitivity

To assess whether the farm environment was protective for the induction of allergies, we used a skin allergy model with primary sensitization with FITC, a skin



Group	Born at	Transferred	Sensitized at
A	AF	No	8 weeks of life
B	Farm	No	8 weeks of life
C	AF	To Farm at 48h of life	8 weeks of life
D	AF	To Farm at 4 weeks of life	8 weeks of life
E	AF	To Farm at 8 weeks of life	12 weeks of life

Fig. 2. Farm environment protects mice for skin allergy. Farm and university animal facility (AF) mice were sensitized on the abdomen with FITC in acetone/dibuthyl-phtalate. Mice were then challenged on one ear with FITC, and on the other ear with the solvent (control ear). Δ ear thickening difference of the challenged ear with the control ear in animals either from the AF (group A) and F2 animals bred at the farm (group B), or mice transferred to the farm at various ages (group C to E). Results represent medians of 12-15 mice/group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

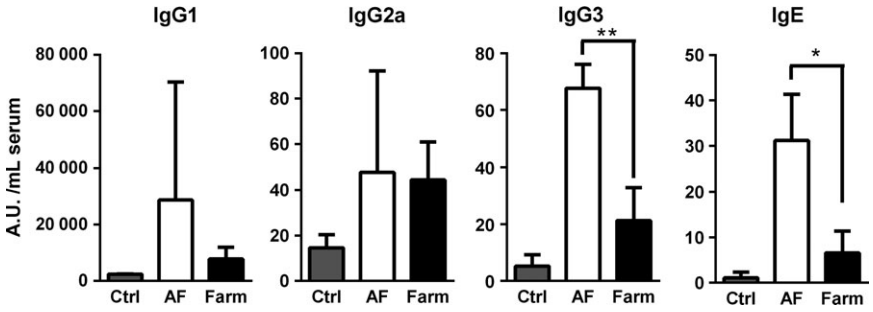


Fig. 3. Titres of serum FITC-specific antibodies. Farm and university animal facility (AF) mice were sensitized on the abdomen with FITC in acetone/dibuthyl-phtalate. Control mice (Ctrl) were not sensitized. Titres of serum FITC-specific antibodies of various isotypes were measured. Results represent mean ± SEM of 12-15 mice/group. Statistical analysis between AF and farm groups, $*P < 0.01$, $**P < 0.001$.

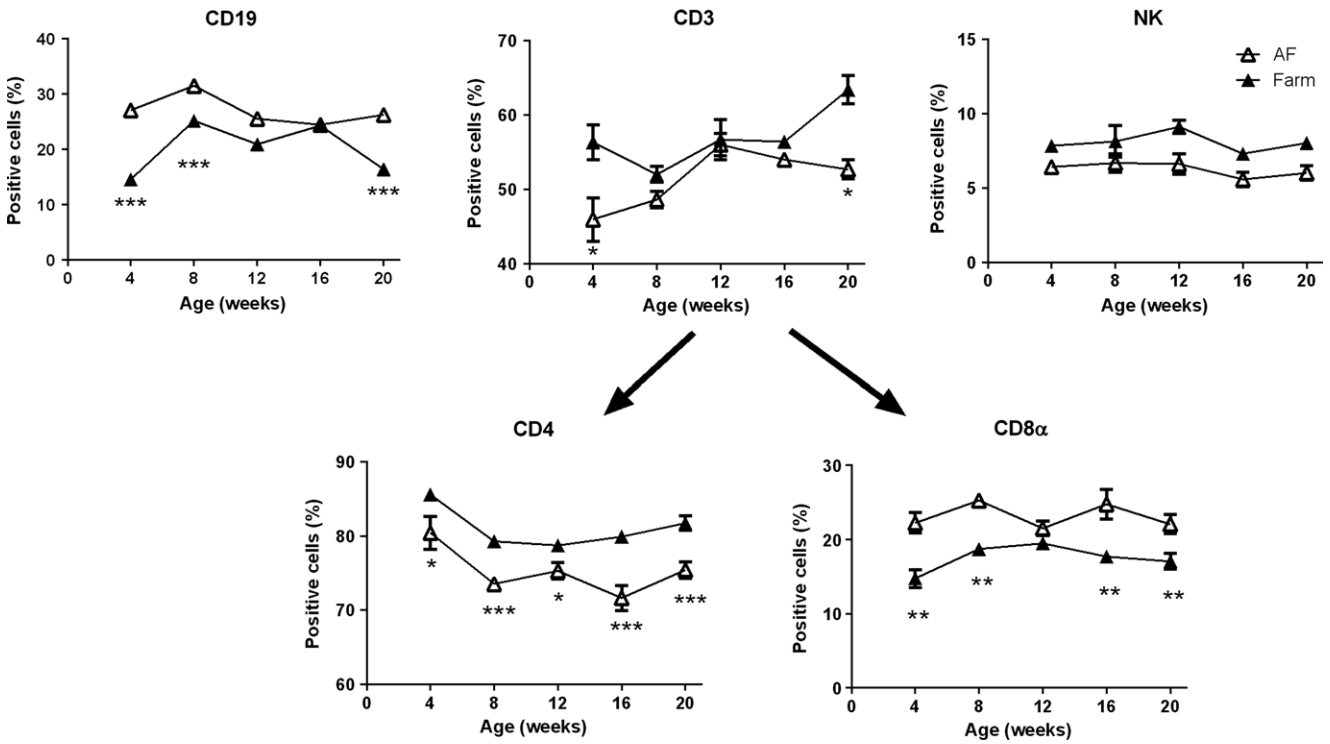


Fig. 4. Proportion of lymphocytes and NK cells in mouse blood. Cells isolated from peripheral blood of university animal facility (AF) (open triangles), and farm (closed triangles) mice were measured every 4 weeks for 20 weeks. The percentage of $CD19^+$, $CD3^+$, NK cells, $CD4^+$ and $CD8\alpha^+$ cells was determined. Results represent the mean ± SEM of 10–15 mice/group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

allergen eliciting a Th2-type response. After initial sensitization to FITC on the mice belly, the skin of one ear was painted with FITC, and inflammation was assessed by measuring ear swelling as compared to the contralateral control ear (Fig. 2). AF mice (group A) presented a clear swelling of the ear with up to threefold increase in ear thickness, when compared to farm mice (group B) ($P < 0.01$). We then explored whether the protection could be conferred to mice that were not bred in farm. The strategy we followed was to transfer mice born in the AF to the farm, either 2 days old pups with their mothers, or 4 or 8 weeks old pups alone. Transfer to the farm at 2 days of life with the mothers followed by a 4 weeks acclimation period gave the same protection than being born in the farm (Fig. 2, group C). Interestingly, mice transferred to the farm at the age of 4 weeks and sensitized at 8 weeks (Fig. 2, group D), and mice transferred at the age of 8 weeks and sensitized at 12 weeks (Fig. 2, group E) were still benefited of some protection; the latter decreasing with animal age at the time of transfer. Systemic sensitization was assessed by measuring FITC-specific antibody titres in mouse serum (Fig. 3). The anti-FITC-specific titres of IgG_3 ($P < 0.001$) and IgE ($P < 0.01$) were lower in farm mice, whereas the anti-FITC IgG_1 titre was lower but statistically non-significant, and the IgG_2 titres remained unchanged. The

presence of circulating antibodies suggests that environmental protection had a systemic impact.

Mice bred in the farm have a specific cell profile in the peripheral blood

To understand immunological changes involved in modulation of tolerance, we first characterized the cell profile of peripheral blood of the two groups. As compared to AF mice, farm mice displayed less $CD19^+$ cells, similar numbers of NK cells, and more $CD3^+$ cells (Fig. 4) suggesting early T cells activation by the farming environment. We then looked at subtypes of $CD3^+$ cells and found that while the percentage of $CD8^+$ cells was lower, the $CD4^+$ cell percentage was increased in the farm mice, suggesting an activation of $CD4^+$ cells in the farm environment. This observation correlated with a tendency to an earlier raise in IgG antibodies in AF mice (not shown). We then used transgenic mice over-expressing FoxP3-GFP to explore the potential activation of regulatory T cells in farm mice. The proportion of $CD4^+CD25^+FoxP3^+$ regulatory T cells was not significantly different between AF and farm mice (Fig. 5a), whereas the proportion of $CD4^+CD25^+FoxP3^-$ activated T cells increased as a function of mouse age in both group of mice but was at all ages significantly higher in farm mice (Fig. 5b). In parallel, the proportion of $CD4^+CD25^-$ naïve T cells decreased in both groups (Fig. 5c) and was significantly lower in farm mice. These data suggest that exposure to cowshed environmental factors early in life favours the expansion of activated T cells.

Activated T cells have an environment-specific pattern of cytokine production

Following the observation that activated T cells were enhanced in farm mice, we assessed the cytokine production pattern of T cells from 4 and 20 weeks old AF and farm mice (Fig. 6). Correlating with anti-CD3/CD28 beads-induced proliferation, IL-2 production was strongly diminished in 4 weeks old farm mice. IL-17 and IL-22 production tended to be enhanced in 4 weeks old farm mice and inhibited in 20 weeks old farm mice, with a statistically significant difference only for IL-22. IL-10 displayed a tendency to be increased in cells of 4 weeks old mice, but was not diminished in 20 weeks old mice. Further measurements including IL-4, IL-5, IL-6, IL-9, IL-13 and IL-18 did not identify notable difference between AF and farm mouse cells (not shown). While IFNγ was present in moderate but similar amounts in both groups (1469.2 pg/mL ± 470.1 in AF; 1524.8 pg/mL ± 2086.3 in the farm) at 4 weeks, cells at 20 weeks produced IFNγ well above the level of detection (> 4000 pg/mL) in both groups.

Characteristics of the gut microbiome from mice living in a farming environment

We were then interested in characterizing the gut microbiota of the various groups of mice, to detect specific microbial triggers for tolerance. Principal coordinate analysis, based on the relative abundance of genera showed that samples defined by age and housing conditions were relatively separated by the first two PCoA first axes which together explain 57.1% total variance (Fig. 7a). The PERMANOVA test confirmed the effect of age in comparisons including AF samples (Fig. 7b) ($P = 0.0359$). PERMANOVA test also showed significant microbiota difference between the two housing conditions in 20-week-old mice (Fig. 7b), while

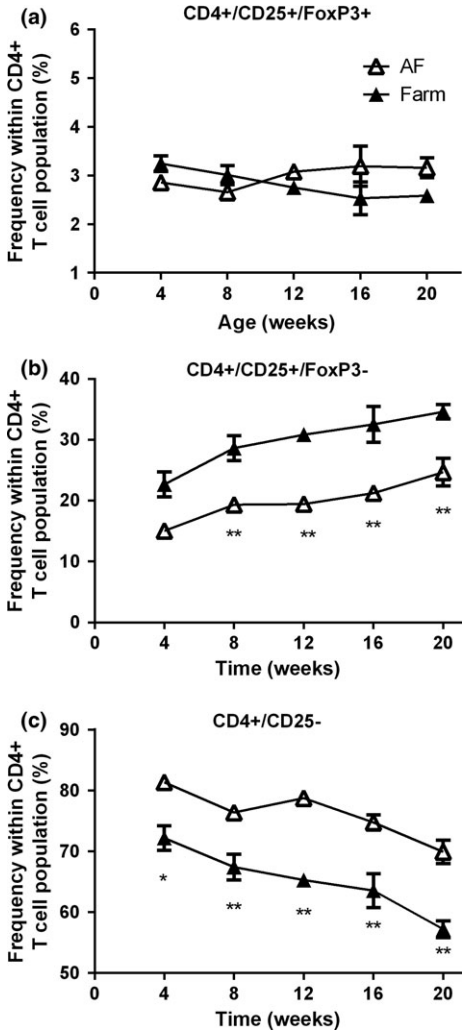


Fig. 5. Activated T cells are enhanced in farm mice. Cells were isolated at 4 weeks or at 20 weeks from peripheral blood of FoxP3-GFP mice bred either at the university facility (AF), or at the farm. Within the population of $CD4^+$ cells, the percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells (a), $CD4^+CD25^+FoxP3^-$ activated T cells (b) and $CD4^+CD25^-$ naïve T cells (C) were determined. Results represent the mean ± SEM of 20 mice from each group. $^*P < 0.05$, $^{**}P < 0.01$.

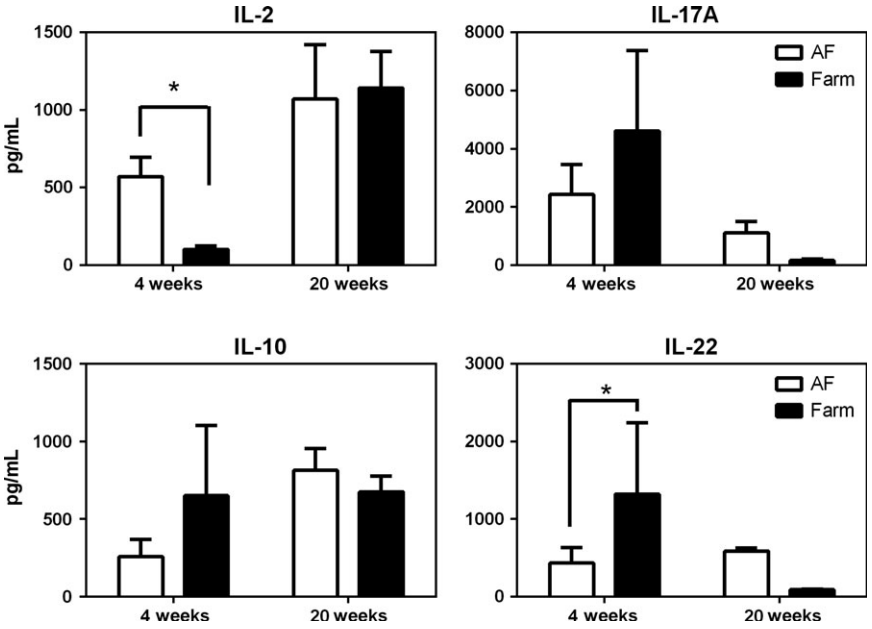


Fig. 6. Cytokine production after activation of spleen T cells from university animal facility (AF) or farm mice. Cells from 4 or 20 weeks old AF mice (white columns) and farm mice (black columns). Cells were activated with anti-CD3/CD28 beads for 3 days, and cytokines were measured in the cell supernatants. The results are presented as mean ± SEM of three independent experiments (each experiment with pooled supernatant from two mice from each group). *$P < 0.05$.

differences observed in the analysis including both groups were close to the significance threshold ($P = 0.0575$). The proportion of several bacterial genera was significantly different in comparisons of groups of samples defined by age or by housing conditions (Fig. 7c). The highest proportion of metagenomic reads corresponding to viruses was identified in the four samples from the 4-week-old farm mice (Table S1). A great majority of these reads (91–95%) were assigned to Mastadenovirus (Table 1). All of the reads mapping to genus Mastadenovirus were classified at the species level as Murine mastadenovirus B. In mice from the three other subgroups (20-week-old farm mice and 4- and 20-week-old AF mice), Mastadenovirus remained undetected and viruses in general had much lower counts (Table 1 and Table S1).

Discussion

Being born and growing up in a farm provides probably the best protection from allergies in young children. Nevertheless, immune mechanisms leading to tolerance are not yet well understood. The breeding of mice in the presence of cowshed directly in a farm reproduces what was first observed in farmers' children and allows exploring some immunological mechanisms. Our results demonstrated that mice born in the barn were less receptive to allergic sensitization in a skin contact allergy model, with a decreased protection if mice were moved later in life to the farming environment. This decreased susceptibility to skin hypersensitivity in these mice correlated with increased circulating activated $CD4^+$ T cell population with an increased proportion of $CD4^+CD25^+FoxP3^-$ activated T cells in mice bred in the farm. The cytokine profile in these mice at 4 weeks of age tended to be skewed towards IL-17 and IL-22 secreting cells, with also an increased in IL-10 secretion. In addition, the presence of a high load of Mastadenoviruses in the gut microbiome of young farm-bred mice identified a potential infectious trigger for allergy protection.

Several epidemiological studies identified the protective effect of the farming environment for allergies in children, and strongly indicated that the microbial environment provided by the farm animals mostly contributed for protection [5, 17]. We were interested to correlate these epidemiologic findings to an experimental model of allergy in a global farming environment. Farm mice were protected from developing an allergic response, as the local swelling observed in case of an inflammatory response was diminished in farm-bred mice. We were also interested to correlate the allergic response to the timing of exposure to the farm, as the specific immune response in farm mice was seen mostly during the 8 initial weeks of life. Thus, mice from different groups (bred at the farm, or moved to the farm at 2 days of life, 4 or 8 weeks of life) underwent skin sensitization and subsequent skin challenge. Timing of exposure to the farming environment was decisive, as mice transferred at a later age to the farm showed more skin allergy than mice born, or transferred early in life

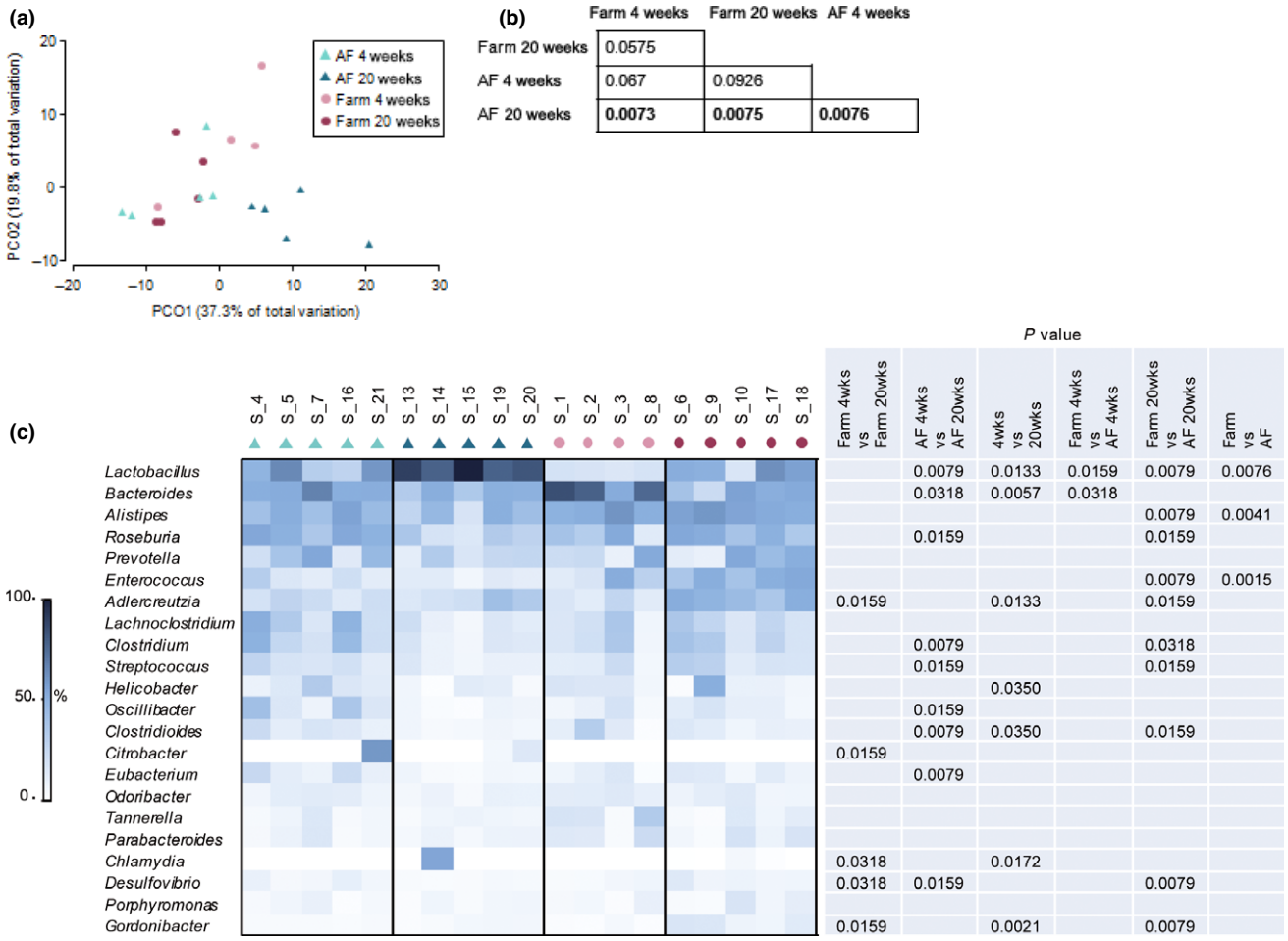


Fig. 7. Similarity between faecal microbial communities. Genera were assigned using CLARK. (a) Principal coordinate analysis based on Bray–Curtis similarity matrix obtained from the relative abundance of bacterial genera. (b) Summary of results of PERMANOVA comparing the four groups of samples. (c) Heat plot showing relative abundance of bacterial genera. Only genera with a mean relative abundance of > 0.6% are presented. Significant changes in relative abundance ($P < 0.05$ by Wilcoxon rank-sum test) are indicated.

to the farm. This observation correlates to the finding in epidemiological studies suggesting that being exposed to the barn already before birth and during the first year of life is most protective [5]. Therefore, we suspected that the farming environment was stimulating the immune system towards a specific phenotype preventing the development of allergy. We were then interested in characterizing the immune profile in the various groups of the mice. Spleen T cells from young farm mice were secreting more IL-10 after activation than T cells from AF mice. In addition to increased IL-10, T cells isolated from young farm mice secreted more IL-17 and IL-22. The farming environment within cowshed contains a high number of Gram-negative bacteria, and increased IL-17 production by cells of farm mice could be explained by exposure to *E. coli* as shown earlier [18]. IL-22 is another cytokine secreted by Th17 cells, which has been linked to gut microbial flora stimulated secretion [19]. Our findings suggest that exposure to microbial environment of the farm in the early life of mice provides a strong stimulus that stimulate CD4+ cells and skews the immune system towards IL-17 and IL-22 producing cells. We also observed an increased IL-10 secretion in farm mice cells potentially providing an allergy protective environment. Most interestingly, this inflammatory pattern disappeared at 20 weeks of age in farm mice, but was not due to T cell anergy, as proliferation after activation was still high in 20 weeks old farm mice. The age-related cytokine profiles could provide an explanation to the studies showing that early exposure to the farming environment in children provides the highest protective effect for allergy [9, 20, 21]. Nevertheless, our data are in contradiction with other findings correlating allergic inflammation in humans with an increase of IL-17 [22, 23]. Murine data provided here should be interpreted only in relation to an early life phenomenon related to allergy protection. We then used transgenic

Table 1. Mastadenovirus load in mouse faecal samples

Sample ID	Housing	Age (weeks)	Ratio (number of reads assigned to Mastadenovirus) /(number of reads assigned to bacteria and archaea)	Ratio (number of reads assigned to Mastadenovirus)/ (number of reads assigned to viruses)
S_1	Farm	4	0.021	0.94
S_2	Farm	4	0.009	0.92
S_3	Farm	4	0.009	0.91
S_8	Farm	4	0.014	0.95
S_6	Farm	20	0	0
S_9	Farm	20	0	0
S_10	Farm	20	0	0
S_17	Farm	20	0	0
S_18	Farm	20	0	0
S_4	AF	4	0	0
S_5	AF	4	0	0
S_7	AF	4	0	0
S_16	AF	4	0	0
S_21	AF	4	0	0
S_13	AF	20	0	0
S_14	AF	20	0	0
S_15	AF	20	0	0
S_19	AF	20	0	0
S_20	AF	20	0	0

AF, animal facility.

mice expressing FoxP3-GFP to studying regulatory and memory T cells. More activated $CD4^+CD45^+FoxP3^-$ cells were found in pups bred in the farm than in those from the AF at all ages. Regulatory T cells $CD4^+CD45^+FoxP3^+$ were overall found in similar numbers at all ages in both groups, although not providing an explanation for the observed differences in clinical and immunologic outcomes between groups. Beura et al. [24] have recently examined the immune system of mice either bred in SPF animal facilities, or from a barn or a pet store environment. They found that the various CD8+ T cell subsets were varying between mice from different environments with CD8+ cells found in lower numbers in AF mice ($CD44^{lo}62L^{hi}$, but not $CD44^{hi}62L^{lo}$).

The gut microbiome has been identified as a potential immunomodulator for allergic diseases. As the farming environment provides a large array of microbial compounds, potentially allergy protective, we characterized the gut microbiome of the various mice colonies. The proportion of several bacterial genera (*Lactobacillus*, *Alistipes*, *Roseburia*, *Adlercreutzia* and *Clostridium*) known to have immunomodulatory properties [25] was significantly different between farm and AF mice. Immunomodulatory properties have in particular been shown for *Lactobacillus Crisptatus* [26], and for *Clostridia* colonization in relation to IL-22 up-regulation in lamina propria lymphocytes [27]. In contrast to AF mice whose microbiome had higher diversity at 4 weeks than at 20 weeks of age ($P = 0.0079$), the Shannon diversity index of farm mice significantly ($P = 0.032$) increased during this time interval from 2.89 (range 1.8–3.18) to 3.97 (range 3.84–4.16). In addition, the analysis of the viral component (DNA viruses) of the gut microbiome showed large number of reads for Mastadenoviruses (a genus including common respiratory and enteric viruses) in young farm mice. Most interestingly, Yamasaki et al. [28] could show inhibition of intestinal anaphylaxis and mucosal tolerance after intraduodenal administration of a replication-defective recombinant adenovirus serotype 40 vector. Whether Mastadenoviruses present in high numbers in farm mice provided a similar trigger remains to be defined.

The main strength of our study was to study the 'global' effect of the farming environment for allergy protection, thus reducing missing specific triggers. This choice nevertheless implied some experimental restrictions, for example technical equipments were limited in the farm not allowing all type of experiments. Also, we did not explore in this study the specific effect of one microorganism; nevertheless, we identified in several bacterial genera and in Mastadenoviruses potential modulators which are worth exploring further. Finally, we were not able to characterize all immune parameters at all times due to animal number constraints, and it remains to be determined whether our immunologic findings are applicable to humans.

Taken together, our study shows that mice bred in a farming environment display a strong protection from allergy, similar to the one that was observed in several epidemiological studies in farmers' children. It also appears that the protective effect is dynamic, as it is mostly effective early in life. This model might be useful to explore specific environmental factors, mostly micro-organisms, in order to decipher the immunological mechanisms and the protective factors involved in 'the farming protective effect'.

Acknowledgements

The authors wish to thank Alain Frossard in Vollèges (Valais, Switzerland) for hosting the mice in his farm, and Myriam Girard for DNA preparation.

Funding

The study was funded by grant #310030-134904 from the Swiss National Science Foundation and the Hans Wilsdorf Foundation.

Conflict of interest

The authors declare no conflict of interest.

References

1 Campbell DE, Boyle RJ, Thornton CA *et al*. Mechanisms of allergic disease – environmental and genetic determinants for the development of allergy. *Clin Exp Allergy* 2015; **45**:844–58.

2 Abrahamsson TR, Jakobsson T, Böttcher MF *et al*. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2007; **119**:1174–80.

3 Perzanowski MS, Ng'ang'a LW, Carter MC *et al*. Atopy, asthma, and antibodies to Ascaris among rural and urban children in Kenya. *J Pediatr* 2002; **140**:582–8.

4 Gassner M. Allergie und Umwelt. *Allergologie* 1989; **12**:492–502.

5 Riedler J, Braun-Fahrländer C, Eder W *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001; **358**:1129–33.

6 Ege MJ, Frei R, Bieli C *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* 2007; **119**:1140–7.

7 Lampi J, Canoy D, Jarvis D *et al*. Farming environment and prevalence of atopy at age 31: prospective birth cohort study in Finland. *Clin Exp Allergy* 2011; **41**:987–93.

8 Lauener RP, Birchler T, Adamski J *et al*. Expression of CD14 and Toll-like receptor 2 in farmers' and nonfarmers' children. *Lancet* 2002; **360**:465–6.

9 Michel S, Busato F, Genuneit J *et al*. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. *Allergy* 2013; **68**:355–64.

10 Lluis A, Depner M, Gaugler B *et al*. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* 2014; **133**:551–9.

11 Debarry J, Garn H, Hanuszkiewicz A *et al*. *Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *J Allergy Clin Immunol* 2007; **119**:1514–21.

12 Bettelli E, Carrier Y, Gao W *et al*. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; **441**:235–8.

13 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**:2114–20.

14 Zhang J, Kobert K, Flouri T *et al*. PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* 2014; **30**:614–20.

15 Ounit R, Wanamaker S, Close TJ *et al*. CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers. *BMC Genom* 2015; **16**:236.

16 Bray R, Curtis J. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr* 1957; **27**:325–34.

17 Braun-Fahrländer C, Riedler J, Herz U *et al*. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; **347**:869–77.

18 Kim OY, Hong BS, Park K-S *et al*. Immunization with *Escherichia coli* outer membrane vesicles protects bacteria-induced lethality via Th1 and Th17 cell responses. *J Immunol* 2013; **190**:4092–102.

19 Satoh-Takayama N, Vosshenrich CAJ, Lesjean-Pottier S *et al*. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 2008; **29**:958–70.

20 Ege MJ, Herzum I, Büchele G *et al*. Prenatal exposure to a farm environment modifies atopic sensitization at birth. *J Allergy Clin Immunol* 2008; **122**:407–12, 412–4.

21 Douwes J, Cheng S, Travier N *et al*. Farm exposure *in utero* may protect against asthma, hay fever and eczema. *Eur Respir J* 2008; **32**:603–11.

22 Chien J-W, Lin C-Y, Yang KD *et al*. Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin Exp Allergy* 2013; **43**:1018–26.

23 Leonardi S, Cuppari C, Manti S *et al*. Serum interleukin 17, interleukin 23, and interleukin 10 values in children with atopic eczema/dermatitis syndrome (AEDS): association with clinical severity and phenotype. *Allergy Asthma Proc* 2015; **36**:74–81.

24 Beura LK, Hamilton SE, Bi K *et al*. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* 2016; **532**:512–6.

25 Asano K, Yoshimura S, Nakane A. Alteration of intestinal microbiota in mice orally administered with salmon cartilage proteoglycan, a prophylactic agent. *PLoS ONE* 2013; **8**:e75008.

26 Tobita K, Yanaka H, Otani H. Anti-allergic effects of *Lactobacillus crispatus* KT-11 strain on ovalbumin-sensitized BALB/c mice. *Anim Sci J* 2010; **81**:699–705.

27 Stefka AT, Feehley T, Tripathi P *et al*. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci* 2014; **111**:13145–50.

28 Yamasaki S, Miura Y, Davydova J *et al*. A single intraduodenal administration of human adenovirus 40 vaccine effectively prevents anaphylactic shock. *Clin Vaccine Immunol* 2013; **20**:1508–16.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Sample description and number of sequence reads at different steps of bioinformatic analysis.