

RESEARCH ARTICLE

Germ-free status and altered caecal subdominant microbiota are associated with a high susceptibility to cow's milk allergy in mice

Bertrand Rodriguez¹, Guénolée Prioult², Rodrigo Bibiloni², Ioannis Nicolis³, Annick Mercenier², Marie-José Butel¹ & Anne-Judith Waligora-Dupriet¹

¹EA 4065, Ecosystème intestinal, Probiotiques, Antibiotiques, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Paris, France; ²Department of Nutrition and Health, Nestlé Research Center, Lausanne, Switzerland; and ³EA 4466, Département Santé Publique et Biostatistiques, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Paris, France

Correspondence: Anne-Judith Waligora-Dupriet, EA 4065, Ecosystème Intestinal, Probiotiques, Antibiotiques, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, 4 avenue de l'Observatoire, 75006 Paris, France. Tel.: +33 1 53 73 99 20; fax: +33 1 53 73 99 23; e-mail: anne-judith.waligora@parisdescartes.fr

Present address: Rodrigo Bibiloni, AgResearch Ltd., Food, Metabolism and Microbiology Section, Ruakura Research Centre, Hamilton 3240, New Zealand.

Received 23 October 2010; revised 10 December 2010; accepted 13 December 2010. Final version published online 19 January 2011.

DOI:10.1111/j.1574-6941.2010.01035.x

Editor: Julian Marchesi

Keywords

Gut microbiota; staphylococci; murine model; germ-free mouse; cow's milk allergy; allergic symptoms.

Abstract

Studies suggesting that the development of atopy is linked to gut microbiota composition are inconclusive on whether dysbiosis precedes or arises from allergic symptoms. Using a mouse model of cow's milk allergy, we aimed at investigating the link between the intestinal microbiota, allergic sensitization, and the severity of symptoms. Germ-free and conventional mice were orally sensitized with whey proteins and cholera toxin, and then orally challenged with β -lactoglobulin (BLG). Allergic responses were monitored with clinical symptoms, plasma markers of sensitization, and the T-helper Th1/Th2/regulatory-T-cell balance. Microbiota compositions were analysed using denaturing gradient gel electrophoresis and culture methods. Germ-free mice were found to be more responsive than conventional mice to sensitization, displaying a greater reduction of rectal temperature upon challenge, higher levels of blood mouse mast cell protease-1 (mMCP-1) and BLG-specific immunoglobulin G1 (IgG1), and a systemic Th2-skewed response. This may be explained by a high susceptibility to release mMCP-1 even in the presence of low levels of IgE. Sensitization did not alter the microbiota composition. However, the absence of or low *Staphylococcus* colonization in the caecum was associated with high allergic manifestations. This work demonstrates that intestinal colonization protects against oral sensitization and allergic response. This is the first study to show a relationship between alterations within the subdominant microbiota and severity of food allergy.

Introduction

Today, the hygiene hypothesis associates perturbations in the gastrointestinal microbiota, due to antibiotic use and excessive hygiene, to the increased prevalence of both allergic and autoimmune diseases (Okada *et al.*, 2010). Indeed, intestinal commensal bacteria and their sequential establishment play a crucial role in the development of gastrointestinal-associated lymphoid tissue and the modulation of the T-helper Th1/Th2/T regulatory balance (Sudo *et al.*, 1997; Gaboriau-Routhiau *et al.*, 2003; Smith *et al.*, 2007; Round & Mazmanian, 2009). Alterations in the sequential bacterial colonization of the gut have been observed in westernized countries (Adlerberth & Wold,

2009) and could therefore be responsible for a Th1/Th2 balance deviation, a major factor in the rise of allergic diseases.

Food allergy affects approximately 5% of young children and 3–4% of adults in westernized countries (Sicherer & Sampson, 2010). Cow's milk allergy (CMA) is the most common allergy in early infancy; its onset may be acute or delayed, and it is responsible for a variety of symptoms and disorders involving the skin and the gastrointestinal tract. CMA can be attributed to either immunoglobulin E (IgE)-mediated, with a disturbance toward a Th2 profile, or non-IgE-mediated mechanisms (Sicherer & Sampson, 2010). Several clinical studies have shown a correlation between allergic diseases and the gut microbiota, with differences in

the composition of bacterial communities in the faeces of children with and without allergic diseases (Penders, 2007). Some authors have associated the presence of the *Clostridium* genus with an increased risk of developing eczema, wheezing, and sensitization (Bottcher et al., 2000; Woodcock et al., 2002; Penders et al., 2006). Several other studies pointed towards quantitative (Bjorksten et al., 1999; Bottcher et al., 2000; Watanabe et al., 2003) and/or qualitative differences in *Bifidobacterium* colonization (Ouwehand et al., 2001; Stsepetova et al., 2007; Gore et al., 2008). However, these microbial investigations have failed to show conclusively that subjects with atopic diseases had altered compositions of the bacterial communities relative to control subjects. In animal models, studies indicated that altering the gut microbiota with antibiotic treatment was linked to a higher susceptibility for peanut allergy (Bashir et al., 2004). However, those studies have not conclusively determined whether gut dysbiosis preceded or resulted from allergic symptoms. In addition, the consequence of sensitization to food antigens on the gut microbiota was seldom studied.

In this study, we investigated (1) whether the gut microbiota impacted the sensitization to a common food allergen and the consecutive allergic response and (2) whether allergic sensitization impacted the composition of the gut microbiota.

Materials and methods

Animals and housing conditions

Germ-free and conventional C3H/HeN mice were purchased at weaning age (21 ± 2 days of life) from Anaxem (INRA, Jouy-en Josas, France) and Charles River Laboratories (CRL, l'Arbresle, France), respectively. Germ-free mice were housed in a sterile isolator and germ-free status

was controlled weekly using standard microbiological methods. Conventional mice were also housed in sterile isolators for protection against the influence of the environment on the microbiota. All mice were allowed *ad libitum* intake of autoclaved tap water and a standard pellet chow that lacked cow's milk proteins (R03, SAFE, Augy, France) and was sterilized by γ -irradiation at 45 kGy. All procedures were carried out in accordance with European guidelines for the care and use of laboratory animals. The protocol was approved by the Regional Council of Ethics for animal experimentation (Ile de France-Paris Descartes – P2.AW.034.07), and all experiments were performed in the technical support animal care facilities of the Institut M dicament Toxicologie Chimie Environnement (IMTCE, Paris Descartes University).

Oral sensitization and immune challenge

For each experiment, conventional and germ-free mice (27–30 per group) were divided into two subgroups of ~ 15 mice each. Oral sensitizations were performed by an intragastric infusion. One subgroup received whey proteins (WP, Lacprodan 80[®], Arla, Lyon, France; 15 mg per mouse) and cholera toxin (CT) as an adjuvant (List Biological, Campbell, CA; $10 \mu\text{g mouse}^{-1}$) in 0.9% NaCl (WP-sensitized groups). The other subgroup received CT alone in 0.9% NaCl (control groups). The sensitizations were performed five times, at weekly intervals, from day 16 to day 44. One week after the last sensitization, on day 51, all mice received an oral challenge with 60 mg of β -lactoglobulin (BLG, Sigma Aldrich) (Fig. 1).

Evaluation of allergic response

Mice were observed and scored 15–45 min after the BLG challenge by two investigators blinded to the sensitization protocol and the mouse groups. Scoring was adapted from

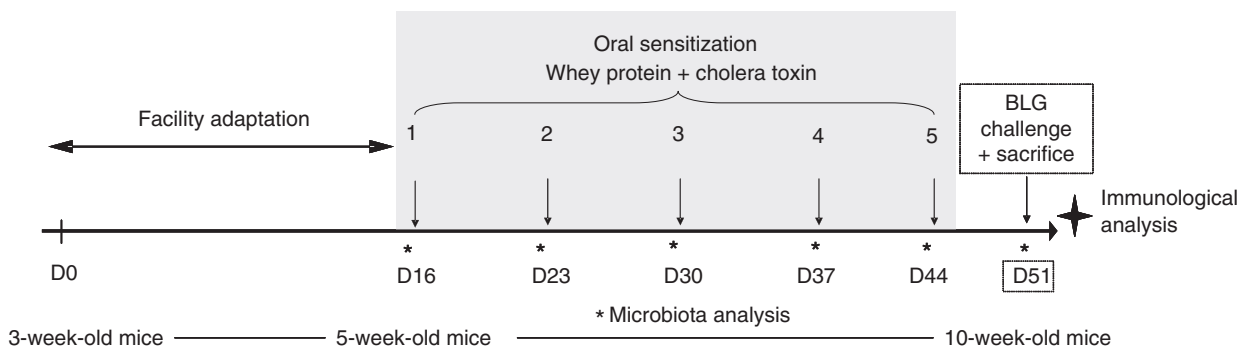


Fig. 1. Mouse sensitization protocol. Three-week-old C3H/HeN mice (germ-free and conventional) were orally sensitized with whey proteins and CT once a week from days 16 to 44 (WP-sensitized mice). Mice were allowed to adapt to the environment for 2 weeks before the first sensitization. Control mice were treated with CT alone. All mice were orally challenged with β -lactoglobulin (BLG) 1 week after the last sensitization. Faecal samples were collected just before each sensitization, and caecal contents were collected on day 51. Allergic responses were assessed on day 51 after the BLG challenge.

Perrier *et al.* (2010). Allergic symptoms were evaluated based on four criteria: a decline in rectal temperature, scratching behaviour, loss of mobility, and puffiness (including bristled fur, oedema around the nose and eyes, laborious breathing). Rectal temperature was recorded before the challenge and after the clinical evaluation. A decline in temperature was graded as follows: $< 3^{\circ}\text{C}=0$, $3\text{--}5^{\circ}\text{C}=2$, and $> 5^{\circ}\text{C}=4$. Scratching was defined as the number of scratching episodes per 15-min interval as follows: 1–3 episodes = 0, 4–5 episodes = 1, and > 6 episodes = 2. Loss of mobility was graded in terms of the duration of absence of any movement as follows: $< 10\text{ min}=0$; $> 10\text{ min}=1$, during the entire trial = 2. Puffiness was graded as none = 0 and puffiness = 2. The clinical score was defined as the sum of the four individual scores, and therefore, ranged from 0 to 10.

Sampling

During the sensitization process, faeces were collected before each sensitization on days 16, 23, 30, 37, and 44. An aliquot was frozen for denaturing gradient gel electrophoresis (DGGE), and another aliquot, for bacterial counting, was immediately resuspended in brain–heart infusion broth with 10% (v/v) glycerol and then frozen. On day 51, after a clinical evaluation, mice were euthanized with an intraperitoneal injection of sodium pentobarbital (CEVA santé animale, Libourne, France). Blood was recovered in K₃-EDTA tubes and plasma was stored at -80°C for immunoglobulin and mouse mast cell protease-1 (mMCP-1) measurements. Spleens were removed and placed in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Fischer-Bioblock, Illkirch, France) for splenocyte culture. Caecal contents were collected on day 51 and processed as described above for faeces.

Detection of total and BLG-specific antibodies in the plasma

Plasma antibody levels were determined by enzyme-linked immunosorbent assay (ELISA).

The total IgE levels were quantified using the OptEIA Set Mouse IgE kit (BD Biosciences, Le Pont de Claix, France), according to the manufacturer's instructions.

Determination of BLG-specific IgE levels was performed by capturing with rat anti-mouse IgE (Pharminogen) antibody, and detecting with freshly prepared biotinylated-BLG (Pierce, Rockford, IL) and streptavidin–HRP (Pierce) (Perrier *et al.*, 2010). Samples were 20-fold diluted and measured in duplicate, and data were expressed in terms of OD_{450 nm}. The levels of anti-BLG IgG1 were determined with BLG as the captured antigen and HRP-labelled-Mab goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) as the detection antibody. Titres were expressed as the log₁₀ of the

reciprocal of the cut-off dilution. The cut-off dilution was the dilution of sample that yielded twice the absorbance of the negative controls. Duplicate wells were run for each sample, and ODs were read at 450 nm.

Measurement of plasma mMCP-1

mMCP-1 was quantified using an ELISA kit (Moredun Scientific Ltd, Penicuik, UK) as described by the manufacturer.

Cytokine production by BLG-stimulated splenocytes

Ex vivo culture of splenocytes was performed as described previously (Menard *et al.*, 2008). Briefly, spleens were gently crushed, filtered through a 70- μm nylon filter (Falcon, VWR, Val de Fontenay, France), and rinsed in RPMI 1640 medium. Splenocytes were treated with Gey's buffer in order to remove red blood cells. Then, splenocytes were rinsed and resuspended in 1 mL of RPMI 1640 containing 25% 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer, 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, 2.5 $\mu\text{g mL}^{-1}$ fungizone, and 10% foetal calf serum. The number of viable cells was determined using the trypan blue dye (0.25%) exclusion method. Splenocytes were adjusted to 2×10^6 cells per well and cultured in 24-well plates with and without 2.5 mg mL⁻¹ BLG at 37°C in a 5% CO₂, 95% air atmosphere (each condition was assayed in duplicate). Culture supernatants were collected after 48 h of culture and stored at -80°C until analysis.

Culture supernatant levels of tumour necrosis factor- α (TNF- α), interleukin-10 (IL-10), IL-4, IL-5 (eBiosciences, Montrouge, France), and interferon- γ (IFN- γ) (BD Biosciences) were quantified using ELISA kits, according to the manufacturer's instructions. The detection limits for ELISAs were as follows: 8 pg mL⁻¹ for TNF- α and IFN- γ ; 4 pg mL⁻¹ for IL-4 and IL-5; and 32 pg mL⁻¹ for IL-10.

Analysis of intestinal microbiota during the sensitization process and after the BLG challenge

PCR coupled with DGGE

For bacterial DNA extraction, 50 mg of faecal material was placed in lysozyme buffer (50 mg mL⁻¹) with 0.3 g of glass beads and disrupted in a bead-beater at maximal speed for 1 min and then incubated at 37°C for 15 min. After centrifugation (10 000 g for 5 min), bacterial DNA was purified from the supernatant with a Qiagen Biorobot EZ1 (Qiagen) according to the manufacturer's instructions.

Bacterial DNA was amplified by PCR with universal bacterial HDA1-GC and HDA2 primers that targeted the V3 region of the 16S rRNA gene (Tannock *et al.*, 2000). The

Table 1. Intestinal microbiota culture media adapted from Butel *et al.* (1998)

Culture media (source)	Bacterial groups or genera quantified
Aerobic genera	
Trypticase soja agar (bioMérieux, Marcy l'Etoile, France) (TS)	Total aerobes
Drigasliki agar (Bio-Rad, Marnes la Coquette, France)	Enterobacteria
D-Cocossel agar (bioMérieux)	Enterococci
Chapman 110 agar (VWR, Strasbourg, France)	Staphylococci
Man Rogosa Sharp agar (Oxoid, Dardilly, France) (MRS)	Lactobacilli
Anaerobic genera	
Columbia agar base+cysteine (160 mg L ⁻¹)+sheep blood (5%)+neomycin (100 mg L ⁻¹)	Total anaerobes
Columbia agar base+cysteine (160 mg L ⁻¹)+sheep blood (5%)+kanamycin (7.5 mg L ⁻¹)+vancomycin (100 mg L ⁻¹)	<i>Bacteroides</i> , <i>Prevotella</i>
Columbia agar base+cysteine (160 mg L ⁻¹)+whole milk (5%)+neomycin (100 mg L ⁻¹)+neutral red (40 mg L ⁻¹)	<i>Clostridium</i>
Wilkins-Chalgren agar base+D-glucose (10 g L ⁻¹)+mupirocin (7.5 mg L ⁻¹)+cysteine (0.5 g L ⁻¹)+Tween 80 (0.5% v/v)	<i>Bifidobacterium</i>
Columbia agar base+cysteine (160 mg L ⁻¹)+sheep blood (5%)+neomycin (100 mg L ⁻¹)+vancomycin (100 mg L ⁻¹)+josamycin (6 mg mL ⁻¹)	<i>Fusobacterium</i>

following amplification program was used: 94 °C for 4 min, 30 cycles consisting of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s; and then 4 min at 72 °C. DGGE was performed as described previously (Bibiloni *et al.*, 2008), and the resulting profiles were compared by determining the Dice similarity coefficient using the BIONUMERICS software package (version 3.0, Applied Maths) at a sensitivity of 2–3%. DGGE was performed on individual faecal and caecal samples and on pools. Pools comprised PCR products from five faecal samples at each time point and for each group (WP-sensitized or control, germ-free, or conventional groups).

Culture method

Faecal and caecal contents were serially diluted and spread on selective and nonselective media using the Spiral System (AES-Chemunex, Bruz, France) and incubated at 37 °C for 24 h under aerobic conditions or at 37 °C for 48 h in an anaerobic cabinet (N₂:CO₂:H₂, 80:10:10, AES-Chemunex). This allowed the isolation, identification, and quantification of aerobic and facultative aerobic bacterial groups, i.e. staphylococci, enterobacteria, enterococci, lactobacilli, and anaerobes, particularly *Bacteroides*, *Clostridium*, *Bifidobacterium*, and *Fusobacterium* (Table 1) (Butel *et al.*, 1998).

Statistical analysis

Results were expressed in terms of the median and were analysed using the Mann–Whitney test. The effects of colonization and BLG-specific IgE levels on mMCP-1 release were analysed using nonparametric ANALYSIS OF COVARIANCE (ANCOVA), with colonization as the main factor and BLG-specific IgE as a covariable. The algorithm used proceeds by

comparison between nonparametric smoothed curves as implemented in the *sm* package for R software (Bowman & Azzalini, 2010). The smoothing coefficient for regression was selected by minimizing the Akaike information criterion. Regression curves were compared for equality between main effect groups and, when the equality hypothesis was rejected, a test of parallelism was further performed. Smoothed curves, equality bands, and parallelism bands were presented (Bowman & Young, 1996). The test results were considered statistically significant when the *P*-values were < 0.05.

Results

Germ-free mice were more responsive than conventional mice to oral sensitization with whey proteins

To investigate the impact of intestinal microbiota on the severity of CMA, germ-free and conventional mice were sensitized with WP+CT and then challenged with BLG. Control mice were treated with CT alone, and then challenged with BLG. In germ-free and conventional mice, the WP-sensitized groups experienced higher allergic symptoms than the control groups (Fig. 2a and b). Allergic symptoms in sensitized mice were accompanied by high levels of blood mMCP-1 (Fig. 2c), high levels of total IgE (Fig. 2d), and BLG-specific antibodies (Fig. 2e and f). Interestingly, among the WP-sensitized mice, germ-free and conventional mice responded differently to the oral challenge with BLG. Although they had similar cumulative clinical scores (Fig. 2a), the rectal temperatures after the BLG challenge were significantly lower (Fig. 2b), and blood mMCP-1 (Fig. 2c) and BLG-specific IgG1 (Fig. 2f)

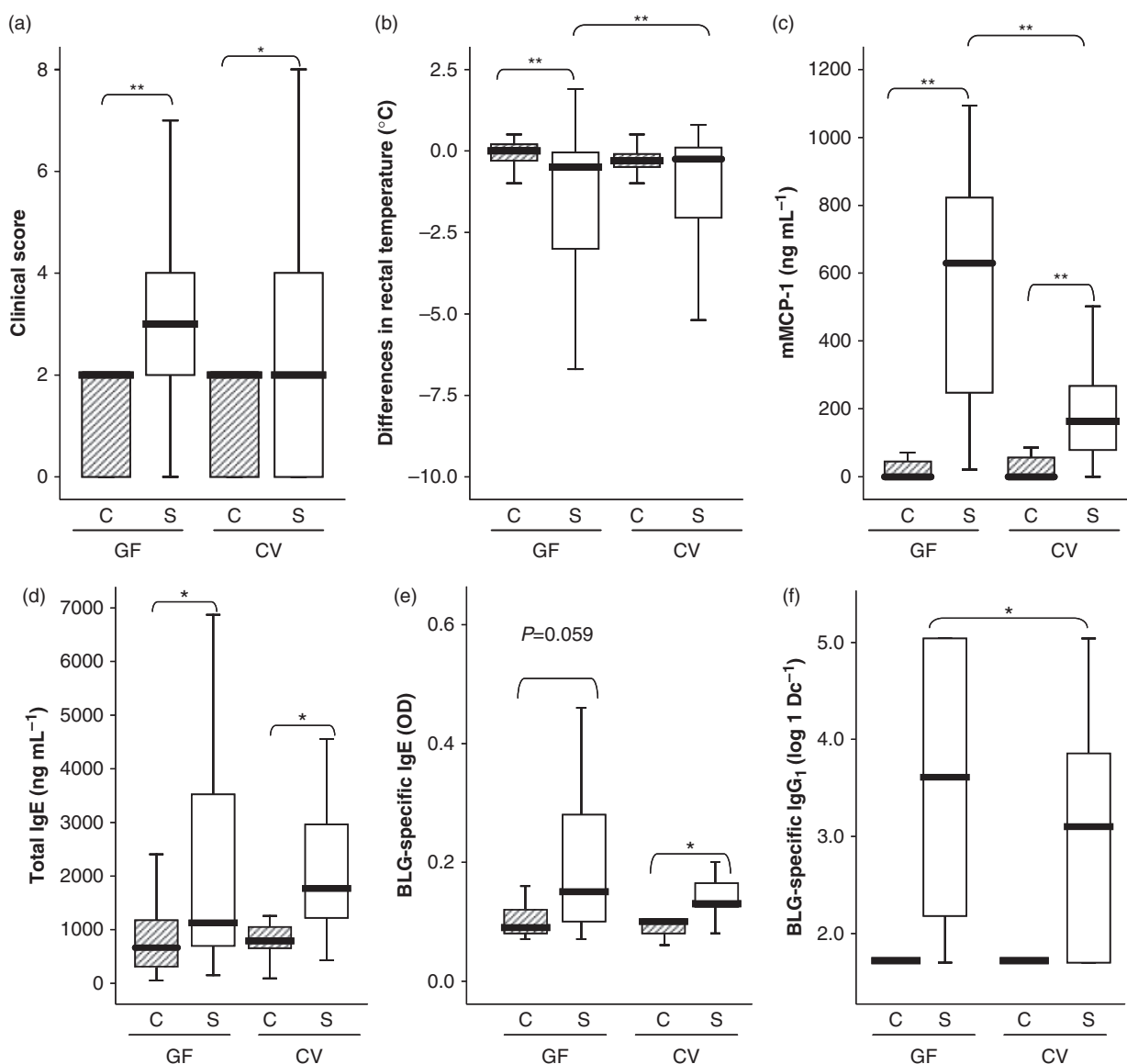


Fig. 2. Allergic response and plasma immunoglobulins in germ-free (GF) and conventional (CV) mice after an oral challenge with β-lactoglobulin (BLG). Mice were sensitized (S; open boxes) with whey proteins and CT; controls (C; hatched boxes) were treated with CT alone. (a) Clinical scores; (b) differences in rectal temperatures (°C) between before and after the BLG challenge; (c) mouse mast cell protease-1 (mMCP-1) levels in plasma (ng mL⁻¹); (d) total IgE (μg mL⁻¹); (e) BLG-specific IgE levels (OD_{450nm}; 20-fold diluted); (f) BLG-specific IgG1 levels (log₁₀ 1/D₅₀; D₅₀, cut-off dilution). The box plots show the median (central horizontal line), the 25th percentile (lower box border), and the 75th percentile (upper box border). The lower and upper horizontal lines show the 10th and 90th percentiles, respectively. **P* < 0.05; ***P* < 0.001.

were significantly higher in germ-free compared with conventional mice. In contrast, the total IgE (Fig. 2d) levels were not different; nevertheless, germ-free mice tended to have higher BLG-specific IgE levels than conventional mice (Fig. 2e).

Because mast cell degranulation is known to be mediated by IgE or IgG1 (Kraft & Kinet, 2007), we were surprised to see that significant differences in mMCP-1 release were observed between WP-sensitized germ-free and conventional mice, whereas BLG-specific IgE levels were not

different between the two groups. Therefore, we investigated the link between mMCP-1 release and BLG-specific IgE in germ-free and conventional mice using ANCOVA. The effect of BLG-specific IgE on mMCP-1 release was significantly different (equality test *P* = 0.04) between germ-free and conventional groups. The two groups showed parallel behaviour (parallelism test *P* = 0.65), suggesting that germ-free mice may release more mMCP-1 than conventional mice in the presence of similar amounts of antigen-specific antibodies (Fig. 3).

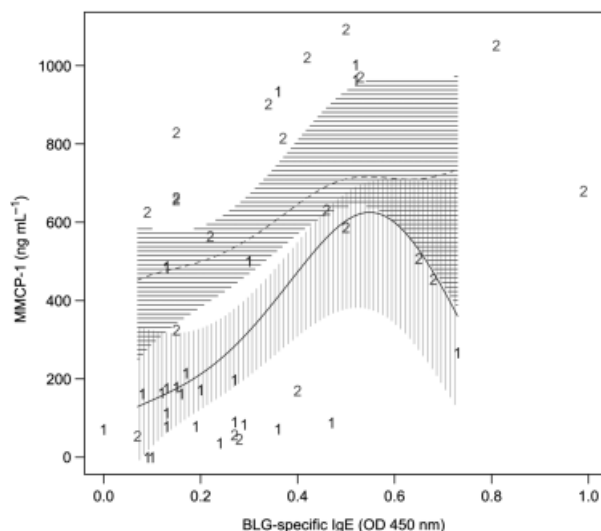


Fig. 3. Nonparametric regressions of mouse mast cell protease-1 (mMCP-1) vs. BLG-specific IgE for conventional (solid line, points labelled 1) and germ-free (dashed lines, points labelled 2) animals. The striped bands indicate the confidence intervals (at a 5% risk level) of the parallelism hypothesis between the two regressions, showing the difference as being due to a vertical shift between two parallel curves.

Splenocytes from sensitized germ-free and conventional mice responded differently to BLG stimulation

We further investigated the differences between germ-free and conventional mice responses to sensitization by stimulating *ex vivo* splenocytes from control and WP-sensitized mice with BLG (Fig. 4). We found that splenocytes from sensitized germ-free and conventional mice responded differently to BLG stimulation. Splenocytes from WP-sensitized germ-free mice produced significantly higher levels of IFN- γ , IL-5, and IL-4 compared with control germ-free mice. No differences were observed in TNF- α and IL-10 levels. Splenocytes from WP-sensitized conventional mice also produced significantly higher levels of IFN- γ and IL-5 compared with control conventional mice. However, they produced higher IL-10 levels, tended to produce higher TNF- α levels ($P = 0.06$), and did not produce detectable IL-4 compared with control conventional mice. In addition, WP-sensitized splenocytes from conventional mice produced significantly higher levels of IFN- γ ($P < 0.05$) and IL-10 ($P < 0.01$) and significantly lower levels of IL-4 ($P < 0.01$) compared with WP-sensitized splenocytes from germ-free mice.

No impact of sensitization on the faecal microbiota composition

To assess the impact of sensitization on microbiota, faecal samples of WP-sensitized mice and control mice were

analysed from day 16 (baseline) to day 44 (end of sensitization) using DGGE and culture methods (Fig. 5). Individual faecal samples and pools of five faecal samples from each group were analysed for each time point. The DGGE method revealed 20–25 bands (i.e. 16S rRNA gene amplicons) per profile. Individual profiles showed over 85% similarity in each group/time point (data not shown). During the sensitization process, all profiles from pooled samples clustered at a node with $> 85\%$ similarity (Fig. 5a), indicating a high level of homology. Similarly, after the sensitization process, individual DGGE profiles of WP-sensitized ($n = 15$) and control ($n = 15$) mice showed approximately 82% similarity (Fig. 5b). The culture method indicated that, on day 44, all WP-sensitized mice were colonized with coagulase-negative staphylococci, enterococci, enterobacteria, lactobacilli, and *Bacteroides* at median levels, respectively, of 4.1, 7.6, 5.2, 9.0, and 6.4 $\log_{10}$ CFU g^{-1} (Fig. 5c). No bacteria of the *Bifidobacterium*, *Clostridium*, or *Fusobacterium* genera, or any other anaerobic genera, were observed (data not shown). No significant differences were seen between day 16 and day 44 and between WP-sensitized and control mice at day 44 for any genera, even though *Bacteroides* levels tended to be lower at the end of the sensitization process (Fig. 5c and d).

Absence or low staphylococci levels in caeca of mice with high allergic scores

A comparison of the microbiota of control and WP-sensitized mice was performed after the challenge with BLG on day 51 (caecum). Individual DGGE profiles of sensitized ($n = 15$) and control ($n = 15$) mice showed approximately 75% similarity (Fig. 6a). This suggests that the individual profiles of mice were unchanged after the BLG challenge. The culture method revealed that all WP-sensitized mice were colonized with enterococci, enterobacteria, lactobacilli, and *Bacteroides* at median levels of 6.0, 3.8, 7.9, and 6.9 $\log_{10}$ CFU g^{-1} , respectively (Fig. 6b), and no differences were observed between control and WP-sensitized mice. Surprisingly, colonization with staphylococci was found to be very variable within the WP-sensitized group compared with control mice (Fig. 6b). Interestingly, while all mice were colonized with staphylococci in the control group, in the WP-sensitized group, only mice that displayed low clinical scores (0–2) were colonized with staphylococci. WP-sensitized mice with high clinical scores (6–8) and experiencing a significant decline of rectal temperature ($> 3^{\circ}C$) were not colonized with staphylococci or were colonized at levels below the detection limit (Fig. 7).

Taken together, these data illustrate that a disturbance in gut microbiota population within the subdominant genera was linked to allergy severity.

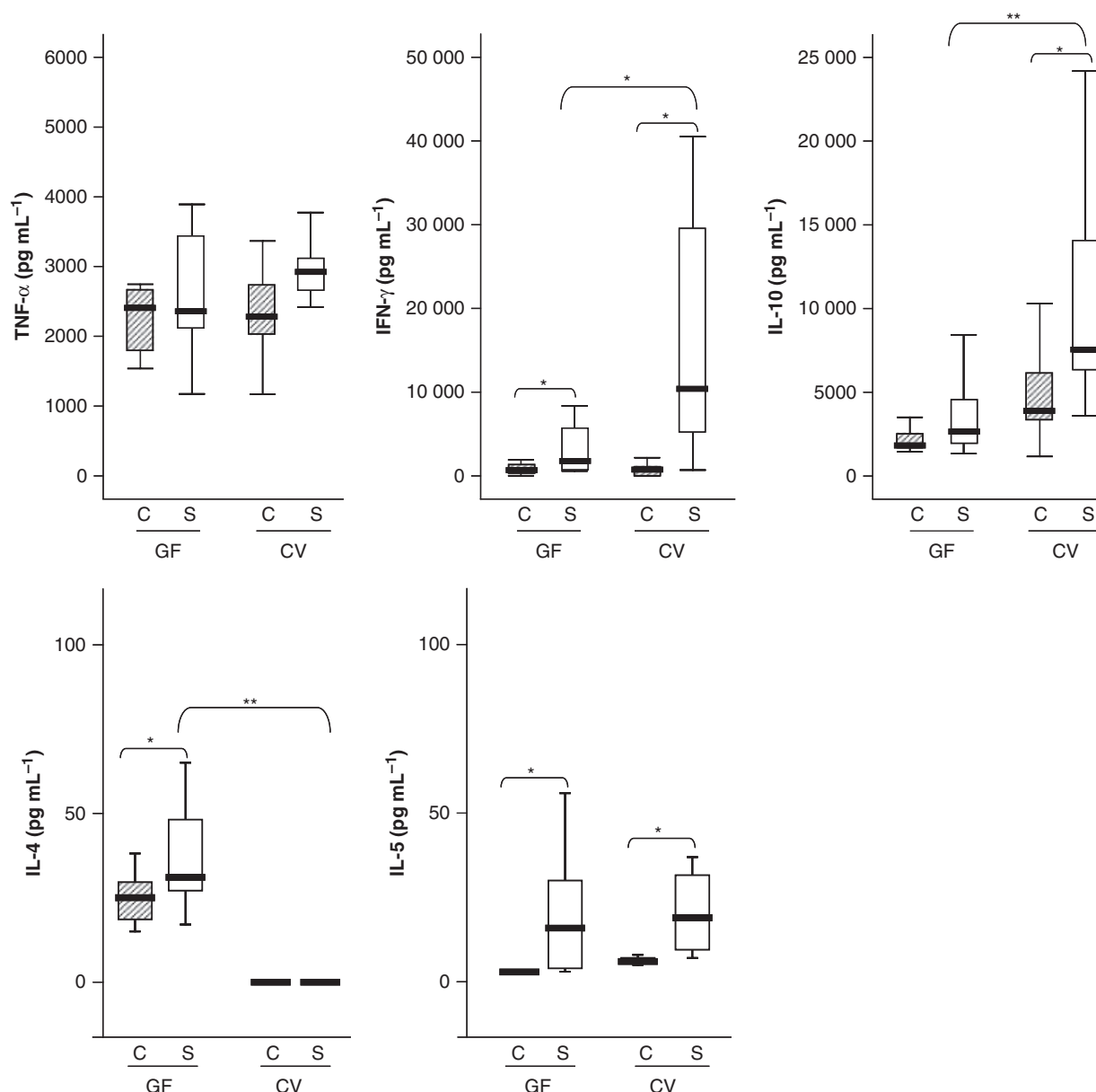


Fig. 4. Cytokine secretion by β -lactoglobulin (BLG)-stimulated splenocytes. Germ-free (GF) and conventional (CV) mice were sensitized (S; open boxes) with whey proteins and CT; controls (C; hatched boxes) were treated with CT alone. Cytokines were measured by ELISA in culture supernatants after a 48-h culture with BLG (2.5 mg mL^{-1}). The box plots show the median (central horizontal line), the 25th percentile (lower box border), and the 75th percentile (upper box border). The lower and upper horizontal lines show the 10th and 90th percentiles, respectively. * $P < 0.05$; ** $P < 0.001$. TNF, tumour necrosis factor; IL, interleukin; IFN, interferon.

Discussion

We investigated the link between gut microbiota and food allergy by comparing the allergic response to cow's milk proteins in germ-free and conventional mice and by assessing the impact of sensitization and allergy on gut microbiota. Germ-free animals are unique models for investigating how the intestinal microbiota shapes the host immune system (Smith *et al.*, 2007). Thus, this model is

relevant for studying the impact of gut microbiota on allergic sensitization (Hazebrouck *et al.*, 2009). In this study, we used a murine model of IgE-mediated hypersensitivity to cow's milk adapted from Li *et al.* (1999). The model consisted of several sensitizations with WP and the mucosal adjuvant CT, followed by an oral challenge with BLG, one of the major allergens in cow's milk. Intragastric sensitizations were preferred over the systemic route (Hazebrouck *et al.*, 2009) to be closer to the human situation.

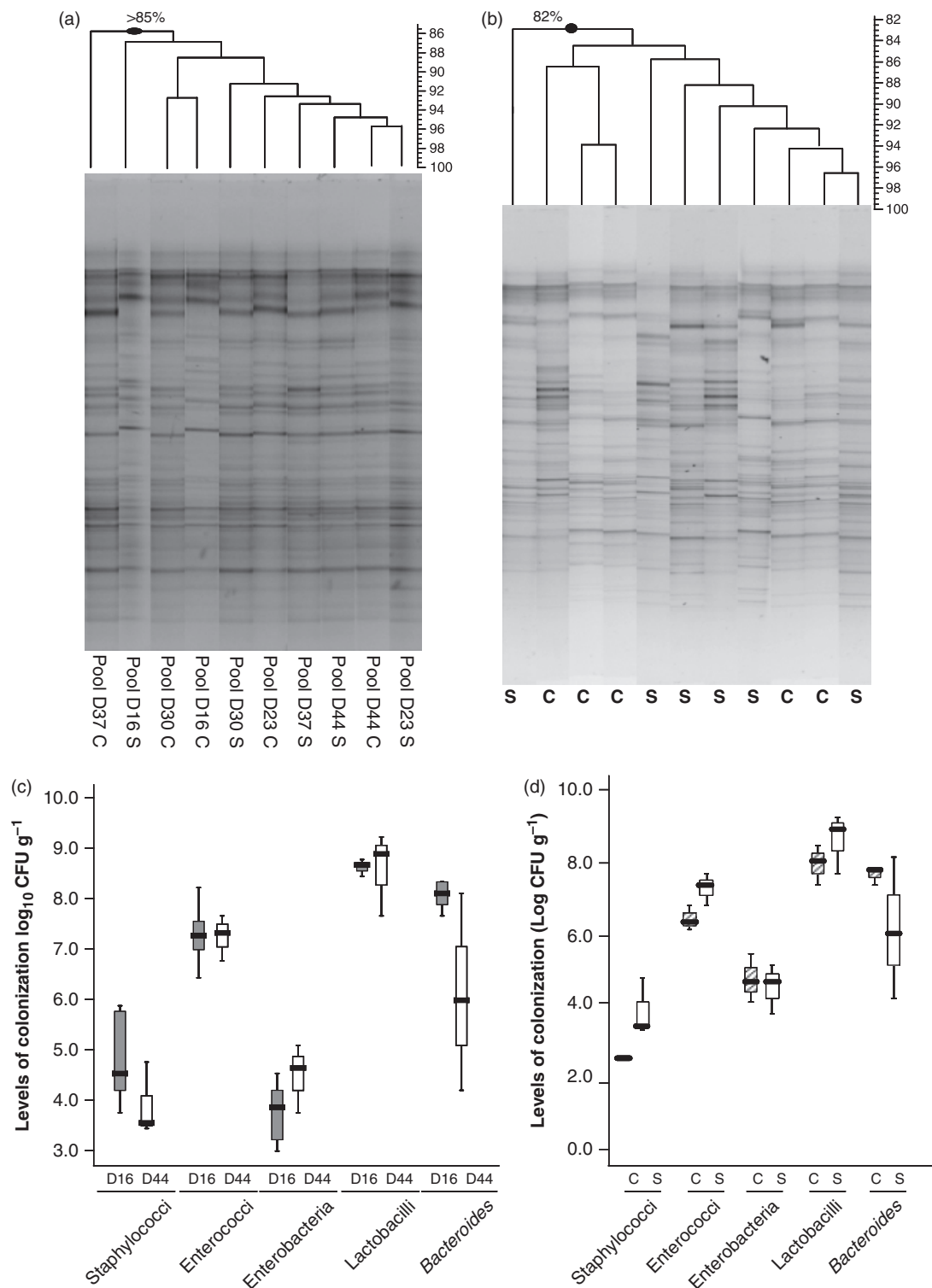


Fig. 5. Faecal microbiota composition during and at the end of the sensitization process. (a, b) Comparison of the dominant microbiota composition between WP-sensitized (S) and control (C) mice using DGGE: (a) PCR products of pooled samples ($n=5$) on days 16, 23, 30, 37, and 44; (b) profiles of individual PCR products on day 44, after the last sensitization. (c, d) Comparison of the dominant and subdominant microbiota using the culture method: (c) in samples collected before sensitization (grey bars, $n=5$) and samples collected after the last sensitization in WP-sensitized mice (open bars, $n=5$); (d) in samples collected after the last sensitization, in control mice (C; hatched bars, $n=5$) and in WP-sensitized mice (S; open bars, $n=5$). The box plots show the median (central horizontal line), the 25th percentile (lower box border), and the 75th percentile (upper box border). The lower and upper horizontal lines show the 10th and 90th percentiles, respectively.

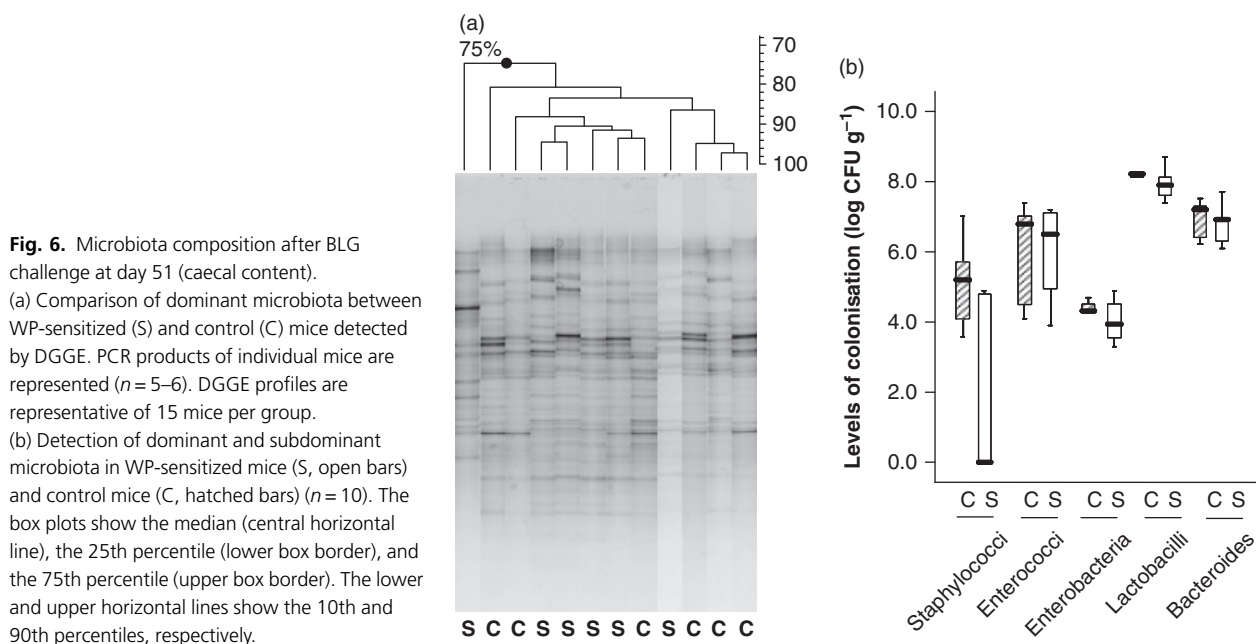
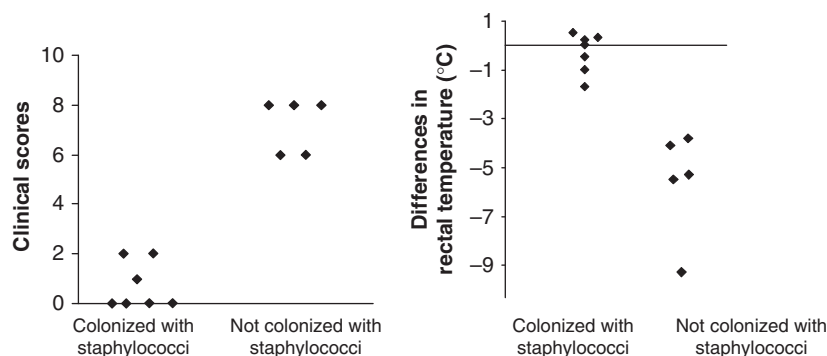


Fig. 7. Clinical scores (left graph) and differences in rectal temperature after BLG challenge (right graph) displayed by conventional sensitized mice colonized or not colonized by staphylococci after BLG challenge.



A decline in body temperature is reported to occur during anaphylaxis both in humans and in mice (Sato *et al.*, 2010). Interestingly, we found that WP-sensitized germ-free mice exhibited a larger decline in rectal temperature upon BLG challenge compared with WP-sensitized conventional mice ($P < 0.05$). They also tended to develop higher clinical scores of allergy than conventional mice, although the difference was not significant. The lack of significant differences may be explained by the variability of allergic responses within each group (presence of low and high responders) and/or the subjectivity of the scoring system for scratching episodes, as described by several authors (Capobianco *et al.*, 2008; Parvataneni *et al.*, 2009; Schouten *et al.*, 2009; Perrier *et al.*, 2010). For a more accurate assessment of the allergic response, we measured allergen-induced degranulation of mast cells by the levels of mMCP-1 release. WP-sensitized germ-free mice released significantly higher levels of mMCP-1 upon BLG challenge than their

conventional counterparts ($P < 0.01$). High mMCP-1 levels in sensitized germ-free mice were accompanied by significantly higher levels of BLG-specific IgG1 in blood ($P < 0.05$).

Taken together, our data illustrated that colonization with a conventional microbiota impacted both sensitization and allergic manifestations. Gut microbiota was previously found to play a role in oral tolerance induction. Oral tolerance to BLG was more effectively induced and maintained in conventional mice colonized with a complete and diversified microbiota than in germ-free mice (Prioult *et al.*, 2003). Gut colonization was shown to improve oral tolerance, particularly when intestinal colonization occurred early in life (Sudo *et al.*, 1997). Others found that the absence of gut microbiota in germ-free mice impaired rather the time course of allergic sensitization than its intensity, with a higher production of IgE and IgG1 in germ-free mice than in conventional ones during the primary immune response, but, in contrast to our results, not at the end of

the sensitization process (Hazebrouck *et al.*, 2009). This discrepancy may be explained by the sensitization route (oral vs. intraperitoneal) and the mouse strain (C3H/HeN vs. Balb/c); both parameters were shown to impact sensitization to food antigens (Tamura *et al.*, 1994).

IgE antibody production and mast cell activity can be modulated by T-cells and their cytokine production (Finkelman *et al.*, 2005). Intestinal colonization can modulate the T-cell population balance and is thus key for the development of a fully functional regulatory T-cell pool (Ostman *et al.*, 2006), as it has been shown in C3H/HeN conventional mice (Gaboriau-Routhiau *et al.*, 2009). Therefore, the protective effect of microbiota could be linked to its impact on the Th1/Th2/regulatory T-cell balance. In this study, BLG-stimulated splenocytes from sensitized conventional mice secreted significantly higher levels of IFN- γ and IL-10 and lower levels of IL-4 than splenocytes from germ-free counterparts. These data suggested that sensitized germ-free mice may have a Th2-skewed immune response and/or a defect in mounting a proper Th1/regulatory T-cell response. The elevated IL-4 levels in WP-sensitized germ-free mice could be involved in the exacerbated allergic response, as shown in a study by Strait *et al.* (2003). The high levels of IFN- γ produced by BLG-stimulated splenocytes from WP-sensitized conventional mice suggest a Th1 bias that may, in part, explain the low levels of specific IgE, IgG1, and mMCP-1 in the plasma (Akdis & Akdis, 2009), inhibit Th2 cells, and, therefore, may play a protective role against allergies (Morafo *et al.*, 2003). In this respect, our conclusions differ from those obtained in a systemic sensitization model (Hazebrouck *et al.*, 2009). Indeed, in the latter study, the production of IL-10 and IFN- γ was enhanced in BLG-stimulated splenocytes from germ-free mice and linked to higher IgE and IgG1 in plasma, and therefore not associated with a protective role against sensitization. Importantly, IL-10 has been reported to downregulate the production of IgE and regulate mast cell maintenance and proliferation in peripheral tissues (Ozdemir *et al.*, 2009). IL-10 can also activate Foxp3⁺ regulatory T-cells, which is associated with a protective effect from respiratory and oral allergy in mice (Lyons *et al.*, 2010). IL-10 is also able to downregulate mast cell Fc ϵ -RI expression (Kashyap *et al.*, 2008; Kennedy *et al.*, 2008). Therefore, the protective impact of gut microbiota in WP-sensitized conventional mice may be driven by IL-10 and its role in mMCP-1 release.

We observed that BLG-specific IgE levels were correlated with mMCP-1 release in both germ-free and conventional mice, as described previously (Schouten *et al.*, 2008). Schouten *et al.* (2008) found that anaphylaxis was positively correlated with specific IgE expression in C3H/HeOuJ mice that developed an IgE-mediated allergy. However, we showed for the first time that, at a given level of BLG-specific IgE, conventional mice released less mMCP-1 than germ-

free mice. This might be explained by the facts that (1) mMCP-1 release can be up- or downregulated by allergen-specific antibodies through their receptors on mast cells (Fc ϵ -RI and Fc γ -RIIB, respectively) (Kraft & Kinet, 2007) and (2) Fc ϵ -RI expression is downregulated by the gut microbiota (Schouten *et al.*, 2009).

Our work highlights the link between gut microbiota and the severity of allergic response. However, we observed variability in allergic symptom development in conventional mice. To investigate whether the sensitization process with CT and BLG affected the composition of gut microbiota, we analysed microbiota using a molecular based-method, which allows studying the dynamics of the dominant bacterial population, and a classical culture technique, which can reveal both the dominant and the subdominant populations. We found that the faecal microbiota were not significantly different before and after sensitization with WP+CT. Furthermore, faecal contents were not significantly different between WP-sensitized and control mice at the end of the sensitization process. Interestingly, using the culture method, similar bacterial colonization was found in WP-sensitized and control mice after the challenge at D51, except for staphylococci. The WP-sensitized mice colonized with staphylococci in caecal content displayed relatively low clinical scores, i.e. 0 and 2. In contrast, mice that were not colonized with staphylococci exhibited high clinical scores (6 and 8). This occurred repeatedly in three independent experiments. This decrease would be the result of the long-term sensitization process rather of the solely clinical response to BLG challenge. Indeed, it would be unlikely that any changes in the microbiota occur within the hour following the BLG challenge. In addition, despite being nonsignificant, some microbiota alterations were observed between D16 and D44. This observation is in accordance with the one of Ouwehand *et al.* (2009), who reported gut microbiota modifications in subjects with allergic rhinitis during the birch pollen season. Besides, *Staphylococcus aureus* in the nasal mucosa were associated with a higher severity of atopic dermatitis (Lebon *et al.*, 2009). In contrast, gut colonization by this species in early childhood was associated with a reduced risk of developing eczema (Lundell *et al.*, 2009). In the present study, colonization with staphylococci was correlated with less severity in the allergic response. It could therefore be linked to a protective effect against allergy or it may be an indicator of alterations in subdominant populations.

To conclude, this is the first study to demonstrate that germ-free mice were more responsive than conventional mice to oral sensitization and challenge with BLG, indicating a protective role of the gut microbiota against food allergy. We also highlight the link between alterations in caecal microbiota and severity of food allergy. The role of staphylococci in allergy merits further investigation.

Acknowledgements

B.R. received grant support from NESTEC. This work was an associated project of the FP7 Marie Curie Actions 'Cross-Talk' ITN project – Grant agreement no. 21553-2. We would like to thank Chantal Martin and Chantal Labellie for helpful assistance in animal experiments. None of the authors had any conflicts of interests.

References

- Adlerberth I & Wold AE (2009) Establishment of the gut microbiota in Western infants. *Acta Paediatr* **98**: 229–238.
- Akdis CA & Akdis M (2009) Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allerg Clin Immun* **123**: 735–746.
- Bashir ME, Louie S, Shi HN & Nagler-Anderson C (2004) Toll-Like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J Immunol* **172**: 6978–6987.
- Bibiloni R, Tandon P, Vargas-Voracka F, Barreto-Zuniga R, Lupian-Sanchez A, Rico-Hinojosa MA, Guban J, Fedorak R & Tannock GW (2008) Differential clustering of bowel biopsy-associated bacterial profiles of specimens collected in Mexico and Canada: what do these profiles represent? *J Med Microbiol* **57**: 111–117.
- Bjorksten B, Naaber P, Sepp E & Mikelsaar M (1999) The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* **29**: 342–346.
- Bottcher MF, Nordin EK, Sandin A, Midtvedt T & Bjorksten B (2000) Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clin Exp Allergy* **30**: 1590–1596.
- Bowman AW & Azzalini A (2010) *R package 'sm': nonparametric smoothing methods* (version 2.2-4). <http://www.stats.gla.ac.uk/~adrian/sm>, http://azzalini.stat.unipd.it/Book_sm
- Bowman A & Young S (1996) Graphical comparison of nonparametric curves. *J Roy Stat Soc C-App* **45**: 83–98.
- Butel MJ, Roland N, Hibert A, Popot F, Favre A, Tess  re AC, Bensaada M, Rimbault A & Szylit O (1998) Clostridial pathogenicity in experimental necrotising enterocolitis in gnotobiotic quails and protective role of bifidobacteria. *J Med Microbiol* **47**: 391–399.
- Capobianco F, Butteroni C, Barletta B, Corinti S, Afferni C, Tinghino R, Boirivant M & Di Felice G (2008) Oral sensitization with shrimp tropomyosin induces in mice allergen-specific IgE, T cell response and systemic anaphylactic reactions. *Int Immunol* **20**: 1077–1086.
- Finkelman FD, Rothenberg ME, Brandt EB, Morris SC & Strait RT (2005) Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J Allerg Clin Immun* **115**: 449–457.
- Gaboriau-Routhiau V, Raibaud P, Dubuquoy C & Moreau MC (2003) Colonization of gnotobiotic mice with human gut microflora at birth protects against *Escherichia coli* heat-labile enterotoxin-mediated abrogation of oral tolerance. *Pediatr Res* **54**: 739–746.
- Gaboriau-Routhiau V, Rakotobe S, Lecuyer E *et al.* (2009) The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* **31**: 677–689.
- Gore C, Munro K, Lay C, Bibiloni R, Morris J, Woodcock A, Custovic A & Tannock GW (2008) *Bifidobacterium pseudocatenulatum* is associated with atopic eczema: a nested case-control study investigating the fecal microbiota of infants. *J Allergy Clin Immun* **121**: 135–140.
- Hazebrouck S, Przybylski-Nicaise L, Ah-Leung S, del-Patient K, Corthier G, Wal JM & Rabot S (2009) Allergic sensitization to bovine beta-lactoglobulin: comparison between germ-free and conventional BALB/c mice. *Int Arch Allergy Imm* **148**: 65–72.
- Kashyap M, Thornton AM, Norton SK, Barnstein B, Macey M, Brenzovich J, Shevach E, Leonard WJ & Ryan JJ (2008) Cutting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. *J Immunol* **180**: 2039–2043.
- Kennedy NS, Barnstein B, Brenzovich J *et al.* (2008) IL-10 suppresses mast cell IgE receptor expression and signaling *in vitro* and *in vivo*. *J Immunol* **180**: 2848–2854.
- Kraft S & Kinet JP (2007) New developments in FcεR1 regulation, function and inhibition. *Nat Rev Immunol* **7**: 365–378.
- Lebon A, Labout JA, Verbrugh HA, Jaddoe VW, Hofman A, van Wamel WJ, van BA & Moll HA (2009) Role of *Staphylococcus aureus* nasal colonization in atopic dermatitis in infants: the Generation R Study. *Arch Pediat Adol Med* **163**: 745–749.
- Li XM, Schofield BH, Huang CK, Kleiner G & Sampson HA (1999) A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immun* **103**: 206–214.
- Lundell A, Hesselmar B, Saalman R, Karlsson H, Lindberg E, Aberg N, Adlerberth I, Wold A & Rudin A (2009) High circulating immunoglobulin A levels in infants are associated with intestinal toxigenic *Staphylococcus aureus* and a lower frequency of eczema. *Clin Exp Allergy* **39**: 662–670.
- Lyons A, O'Mahony D, O'Brien F *et al.* (2010) Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy* **40**: 811–819.
- Menard O, Butel MJ, Gaboriau-Routhiau V & Waligora-Dupriet AJ (2008) Gnotobiotic mouse immune response induced by *Bifidobacterium sp.* strains isolated from infants. *Appl Environ Microb* **74**: 660–666.
- Morafo V, Srivastava K, Huang CK, Kleiner G, Lee SY, Sampson HA & Li XM (2003) Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *J Allerg Clin Immun* **111**: 1122–1128.
- Okada H, Kuhn C, Feillet H & Bach JF (2010) The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol* **160**: 1–9.
- Ostman S, Rask C, Wold AE, Hultkrantz S & Teleme E (2006) Impaired regulatory T cell function in germ-free mice. *Eur J Immunol* **36**: 2336–2346.

- Ouwehand AC, Isolauri E, He F, Hashimoto H, Benno Y & Salminen S (2001) Differences in *Bifidobacterium* flora composition in allergic and healthy infants. *J Allergy Clin Immunol* **108**: 144–145.
- Ouwehand AC, Nermes M, Collado MC, Rautonen N, Salminen S & Isolauri E (2009) Specific probiotics alleviate allergic rhinitis during the birch pollen season. *World J Gastroenterol* **15**: 3261–3268.
- Ozdemir C, Akdis M & Akdis CA (2009) T regulatory cells and their counterparts: masters of immune regulation. *Clin Exp Allergy* **39**: 626–639.
- Parvataneni S, Gonipeta B, Tempelman RJ & Gangur V (2009) Development of an adjuvant-free cashew nut allergy mouse model. *Int Arch Allergy Immun* **149**: 299–304.
- Penders J (2007) The role of the intestinal microbiota in the development of atopic disorders. *Allergy* **62**: 1223–1236.
- Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, Adams H, van RR & Stobberingh EE (2006) Gut microbiota composition and development of atopic manifestations in infancy: the KOALA birth cohort study. *Gut* **56**: 661–667.
- Perrier C, Thierry AC, Mercenier A & Corthesy B (2010) Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* **40**: 153–162.
- Priault G, Fliss I & Pecquet S (2003) Effect of probiotic bacteria on induction and maintenance of oral tolerance to beta-lactoglobulin in gnotobiotic mice. *Clin Diagn Lab Immunol* **10**: 787–792.
- Round JL & Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* **9**: 313–323.
- Sato Y, Akiyama H, Matsuoka H et al. (2010) Dietary carotenoids inhibit oral sensitization and the development of food allergy. *J Agr Food Chem* **58**: 7180–7186.
- Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE & Garssen J (2008) Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *Int Arch Allergy Immun* **147**: 125–134.
- Schouten B, van Esch BC, Hofman GA, van Doorn SA, Knol J, Nauta AJ, Garssen J, Willemsen LE & Knippels LM (2009) Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. *J Nutr* **139**: 1398–1403.
- Sicherer SH & Sampson HA (2010) Food allergy. *J Allerg Clin Immunol* **125**: S116–S125.
- Smith K, McCoy KD & Macpherson AJ (2007) Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol* **19**: 59–69.
- Strait RT, Morris SC, Smiley K, Urban JF Jr. & Finkelman FD (2003) IL-4 exacerbates anaphylaxis. *J Immunol* **170**: 3835–3842.
- Stsepetova J, Sepp E, Julge K, Vaughan E, Mikelsaar M & De Vos WM (2007) Molecularly assessed shifts of *Bifidobacterium* ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. *FEMS Immunol Med Mic* **51**: 260–269.
- Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C & Koga Y (1997) The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* **159**: 1739–1745.
- Tamura S, Shoji Y, Hasiguchi K, Aizawa C & Kurata T (1994) Effects of cholera toxin adjuvant on IgE antibody response to orally or nasally administered ovalbumin. *Vaccine* **12**: 1238–1240.
- Tannock GW, Munro K, Harmsen HJ, Welling GW, Smart J & Gopal PK (2000) Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl Environ Microb* **66**: 2578–2588.
- Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y & Kumemura M (2003) Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* **111**: 587–591.
- Woodcock A, Moradi M, Smillie FI, Murray CS, Burnie JP & Custovic A (2002) *Clostridium difficile*, atopy and wheeze during the first year of life. *Pediatr Allergy Immunol* **13**: 357–360.