

IMPACT OF INTESTINAL MICROFLORA ON ALLERGY DEVELOPMENT (ALLERGYFLORA)

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FINAL REPORT

Table of contents

1	Introduction	2
2	Materials and methods	4
3	Results	13
3.1	Method validity	13
3.2	Method compability between centres	17
3.3	Clinical outcome	21
3.4	Intestinal colonization patterns	28
3.5	Strain turn-over	33
3.6	Development of T-RFLP for genetic assessment of intestinal microflora	35
3.7	Allergy development in relation to colonization pattern	38
4	Discussion	41
5	Conclusion	45
6	References	46

Figure 1

Appendix

1. Introduction

Allergy denotes "altered reactivity". The allergic individual mounts inappropriate immune responses towards harmless environmental substances that have been present in the environment for thousands of years. Allergies are, thus, the result of a faulty programming of the immune system, the cause of which is still unknown.

The tendency to develop allergy is determined in early childhood as shown by adoption studies (Hjern et al., 1999). Many observations suggest that frequent exposure to microbes is protective against allergy development. Allergy is more common in children who have no or few elder siblings than among children born and raised in large families (Strachan, 1989; von Mutius et al., 1994a) and day care from early age protects against allergy development (Kramer et al., 1999; Ball et al., 2000). Children who grow up on a farm with livestock, or in families that keep pets, develop allergies less often than children who have not been exposed to animals during their first years of life (Riedler et al., 2001; Hesselmar et al., 1999). Allergies are more common in Western market economies than in the former socialist countries in Eastern Europe (von Mutius et al., 1994; Bråbäck et al., 1994) and within a country, allergies are more common among the wealthy than among the poor (Strachan, 1989). All these observations fit with the "hygiene hypothesis" as originally formulated in 1989 (Strachan, 1989). This hypothesis states that microbial stimulation of the immune apparatus is necessary for its maturation and proper function and that lack of such stimulation results in allergy development (Strachan, 1989).

The commensal microflora contains the vast majority of microbial antigens confronting the developing immune system. Exposure to microbes via the gut may be a key determinant in proper development of tolerance to innocuous antigens. This is supported by the observation that persons who have been infected with fecal-orally spread pathogens such as hepatitis A, *Helicobacter pylori* and *Toxoplasma gondii*, have less allergies than individuals of the same age who have not had such infections in childhood (Matricardi et al., 2000). In contrast, common viral infections, such as measles and rubella, seem not to influence the risk of developing allergies (Matricardi et al., 2000). Furthermore, children who develop allergies exhibit delayed maturation of the secretory immune system compared with children who do not develop allergies (Payette et al., 1977; van Asperen et al., 1985; Neffen et al., 1986). Secretory IgA responses correlate with the degree of environmental microbial exposure (Mellander et al., 1985)

It is logical that the intestinal milieu influences immune reactivity to environmental antigens. All antigens that are either inhaled or swallowed sooner or later end up in the gut and become processed by the gut-associated lymphoid system. Innocuous antigens which are presented via the gastro-intestinal route normally induce a state of immunological tolerance, so called oral tolerance (Mestecky et al., 1996). Oral tolerance is manifested by suppressed immune responsiveness to the same antigens when later administered systemically. Both so called Th1 and Th2 responses are suppressed (Mowat et al., 1996). Oral tolerance has been described in a range of animal species, as well as human beings (Mestecky et al., 1996). Animals raised under germfree conditions have lower capacity to develop tolerance to dietary antigens compared to conventional animals, while certain bacterial toxins, notably cholera toxin and *E. coli* heat labile toxin, counteract oral tolerance (Moreau et al., 1988; Gaborieau-Routhiau et al., 1996; Sudo et al., 1997). Which types of microbes that are needed for proper oral tolerance induction, and the mechanism by which they affect maturation of tolerance processes, is not known.

Intestinal bacteria may impact on tolerance induction in several ways. First, bacteria may influence how intestinal epithelial cells function and thereby how foreign antigens are handled. Secondly,

bacteria and/or bacterial products from the commensal microflora can be taken up across the mucosal barrier and influence antigen-presenting cells in the Peyer's patches and intestinal *lamina propria*. As recent research has shown that Gram-positive and Gram-negative bacteria have different effects on antigen-presenting cells (Hessle et al., 2000; Hessle et al., 2003; Karlsson et al., 2004, Smits et al., 2004), the composition of the commensal flora may affect antigen handling in the gut-associated lymphoid system. Thirdly, intestinal bacteria probably represent a significant proportion of all antigens which the immune system encounters. Thus, the degree of strain turn-over in the commensal ecosystem will determine the degree of immune stimulation of the individual. The complex interaction between commensal microbes and the host calls for detailed and meticulous studies of the composition of the gut flora in relation to allergy development.

A role for the intestinal flora in determining the risk for allergy development is suggested by theoretical models of the function of the gut-associated lymphoid system and tolerance mechanisms, as outlined above. Furthermore, there are epidemiological associations between microflora composition and hygienic living conditions. For example, colonization by *E. coli* is delayed in Swedish infants as compared to Pakistani infants (Adlerberth et al., 1991). Furthermore, the turn-over of individual strains of *E. coli* in the microflora is also low in Swedish infants, compared with infants from developing countries (Adlerberth et al., 1988). This might imply that infants in highly industrialized countries encounter an overall lower number of microbial antigens compared to infants in developing countries. In accordance, Swedish infants exhibit a delayed maturation of the salivary secretory IgA response, compared to Pakistani infants (Mellander et al., 1985). As mentioned before, children who develop allergy often exhibit a transient "IgA deficiency" at mucosal sites, which might be a sign of an overall low immune stimulation by mucosal microbes.

Since the formulation of the hygiene hypothesis in 1989, the association of allergy with small families and firstborn children has been repeatedly confirmed. More recent observations of a reduced allergy risk in children living on farms or exposed to pets provide indirect corroboration of the hypothesised link between infection and allergy. However, direct evidence of a protective effect from specific infections remains elusive. It is now essential to identify which type of microbial stimulation that protects us from exaggerated responses to common environmental antigens, including allergy. Our hypothesis is that a deficient commensal microflora in infancy fails to support the development and maturation of regulatory immunological mechanisms, allowing exaggerated responses to environmental antigens to occur. It is also important to study whether exposure to some microbes may instead increase the risk of developing allergic sensitization.

The microflora could be deficient in three alternative, but not mutually exclusive, ways:

- certain key groups of microorganisms able to facilitate maturation of regulatory mechanisms could be absent or severely reduced in the microflora of contemporary infants,
- other types of bacteria which have replaced "traditional" microbial groups may impede tolerogenic presentation of innocuous antigens, and/or
- a generally reduced turn-over rate of microbial strains in the infants' intestinal microflora provides too weak a stimulus to the developing immune system for e.g. regulatory T cells to develop and/or expand.

2. Materials and methods

Overview

Infants were enrolled at delivery (Rome, London) or the parents-to-be were enrolled at the maternity clinic (Göteborg). At entry into the study, baseline variables were recorded in questionnaires, such as family size and structure, housing conditions, etc. A sample of house dust was collected and analyzed for aeroallergens. During the course of the study, the parents registered feeding practices, vaccinations, type of day care, illnesses and medication in a record book kept in their homes.

The intestinal microflora was assessed by semiquantitative culture of a rectal swab by three days of age and by quantitative culture of faecal samples at 1, 2, 4 and 8 weeks of age and at 6 and 12 months of age. Faecal samples were also frozen for later assessment by non-culture dependent techniques developed as part of the project.

At 18 months of age, children were invited to attend a clinical examination by a paediatrician. Blood was collected for measurement of total and specific serum IgE and clinical allergy was diagnosed by anamnesis and clinical status.

The project was scheduled to include 300 infants, 100 each from Göteborg, London and Rome. The results presented here are based on 116 infants recruited in Göteborg, 108 in London and 100 in Rome. Data from the 18 month visit are available for all of the children in Göteborg, 106 in London, but, at the time of the statistical analysis, only 79 children in Rome had been examined at age 18 months. Clinic visits in Rome continued throughout November 2004 to February 2005 and the analysis will be updated in due course. (NB. In our application, we anticipated that data analyses at this stage would be based on only 50 infants per centre, and this figure has been exceeded in all three centres.)

Recruitment of infants and assessment of background data

In Göteborg, parents-to-be were recruited at the maternity clinic. Inclusion criteria were normal pregnancy without complications, and expected vaginal delivery at term (at least 38 weeks' gestation). In order to increase recruitment and compliance, atopic parents were mainly targeted, but a smaller group of non-atopic families were also included.

In Rome and London, the parents were contacted in the obstetric department in the first day after delivery by a doctor or a nurse. Exclusion criteria were twin birth, antenatal problems, language difficulties, congenital malformations and prematurity (<37 weeks gestation).

A questionnaire was administered at the 3rd day of life (Rome, London) or antenatally (Göteborg) to parents addressing the following items: 1) Father's and mother's profession, birth date, occupation, ethnic background, family structure; 2) mother's and father's atopy: doctor's diagnosis of asthma, eczema, allergic rhinoconjunctivitis; active disease in the last 12 months; 3) family environment: mother's smoking during pregnancy, mother or father currently smoking, pets inside the home during pregnancy or currently; mould in the house.

Obstetric information was collected from the parents after delivery and included: gestation, time of delivery, type of delivery, duration of ruptured membranes, water birth, antibiotics given to the mother (type and duration) and to the baby (type), maternal high vaginal swab and results.

Diary cards (weekly for the first 2 months, monthly for the 3rd up to the 12th month) were given to the parents to facilitate recording of relevant events in the 1st year of life of their child. The diary addressed the following issues: 1) baby's health: antibiotics and other prescriptions, cradle cap, eczema, nappy rash, other rash, colic, diarrhoea, constipation, vomiting, "cold", wheezing, temperature; 2) contact with health care team: GP/casualty consultation, vaccinations; 3) food intake: introduction of new food (including yoghurt/probiotics); cessation of breast feeding; new pets in the house. The parents reported suspected allergic symptoms in the infants to the study team and the infant were referred for examination to one of the pediatricians in the team.

A questionnaire was administered to parents at 6 and 12 months of age addressing the following items: 1) feeding: breast-feeding, mother's diet, bottle-feeding (starting time, duration, bottle sterilization method, formula used), use of dummy or pacifier and methods of cleaning, introduction of solid foods, childcare; 2) home environment: child's and parents' bed, time spent outdoor, animals inside and outside the home, smoke, moving house, renovation or painting the home, dampness; 3) medications, with a special focus on antibiotics.

The questionnaire included also items on health problems: 1) breathing problems: sneezing, runny or blocked nose, wheezing or whistling in the chest, dry cough at night apart from infections, their onset, triggers and recurrence, doctor's diagnosis and drug prescriptions; 2) skin problems: itchy rash, its location/distribution, sequence of appearance, and drug prescriptions; 3) gastrointestinal problems: including infantile colic, abdominal pain, vomiting and nausea, diarrhoea and constipation; 4) food-related problems: their quality, the suspected offending food stuff, doctor's diagnosis, diet prescriptions, growth disturbances.

Protocols for clinical examinations and questionnaires were harmonized among the partners in the ALLERGYFLORA project. During the initial calibration phase all the questionnaires and other forms were translated during preparatory meeting to ensure that these and the schedules for recruitment and visiting the families etc. were adjusted to suit the conditions of the regions where the study was performed. Local environmental conditions such as infant feeding habits have been discussed and relevant questions have been added into the questionnaires. Schedules for visits and patient handling were adjusted and harmonized between the centers.

Microbiological analyses

Microbiological analyses were performed using the same methodology in all three centres.

The three laboratories used the same reagents (media components, anaerobic culture systems, typing kits, etc.) purchased from the same manufacturers. Further, all personnel that perform microbial analyses were trained in the laboratory of the coordinating centre, in order that sample handling, culture and identification of the microbes be completely uniform. A method transfer and calibration phase of six months was devoted to this work. Further, quite frequent meetings were held during the course of the project involving not only the researchers, but also all personnel involved in the practical work, including nurses, technicians and paediatricians. This facilitated identifications of small details in

procedures that needed to be harmonized. We have found that direct communication between technicians, study nurses and clinicians in the different centres was a key factor for successful integration of the microbiological project.

The extensive effort devoted to this transfer and calibration of methodology in all details, resulted in inter-laboratory variability in the same range as the intra-laboratory variability inherent to all microbial culture methodology (see Results section).

A common database in the EpiInfo system was developed for entry of microbiological and clinical data and was used by all partners participating in the clinical part of the project, enabling pooling of the data and analysis as a common cohort.

Sampling of the intestinal microflora

A sample of the rectal flora was obtained 3 days after delivery using a cotton-tipped swab. The swab was put in COPAN's transportation medium and transported to the laboratory where it was cultured within 24 h after collection.

Faecal samples were obtained at 1, 2, 4 and 8 weeks and at 6 and 12 months of age. Freshly voided faeces were collected by the parents and a sample was placed in a sterile, vented Petri dish using a sterile wooden spoon, which was then placed in a gas-proof plastic bag in which an anaerobic atmosphere was generated (AnaeroGen Compact, Oxoid Ltd, Basingstoke, Hampshire, England). Anaerobiosis was confirmed by inclusion of an anaerobic indicator in the bag (BR55, Oxoid Ltd). The samples were kept refrigerated until being transported to the laboratory, where they were processed within 24 h after collection.

Bacterial culture

Rectal swab samples were cultured on selective and non-selective media for the isolation of aerobic or facultatively anaerobic bacteria (Table 1). The inoculum was spread with a microbiological loop to obtain single colonies.

For faecal samples, an initial 10^{-1} dilution was prepared by homogenising 150 mg of faeces obtained with a calibrated measuring spoon in 1.5 mL of sterile buffered peptone water. This dilution was further diluted logarithmically in sterile buffered peptone water from 10^{-2} to 10^{-9} . Appropriate dilutions were spread on selective and non-selective media for the isolation of anaerobic and aerobic or facultatively anaerobic bacteria (Table 1).

Anaerobic cultures were performed for three days in anaerobic jars using the BBL GasPak anaerobic system (Becton Dickinson Microbiology Systems, Sparks, USA). The agar plates were pre-reduced for at least 24 h prior to use. For the isolation of spore-formers (clostridia), a portion of the 1:10 sample dilution was mixed with equal proportions of 99% ethanol and incubated on a shaker at room temperature for 30 min. After this treatment, which kills vegetative cells, the sample was further diluted, plated on Brucella blood agar plates and incubated as described above.

From appropriate dilutions on selective and non-selective agar plates, single colonies of different morphology were separately enumerated, Gram-stained, and subcultured for further identification. All isolates were frozen at -70°C in Hogner's freezing medium.

Table 1. Media and culture conditions used for different bacterial groups.

Bacterial group	Culture medium (agar)	Conditions	Time (days)
Total aerobes and facultatives	Colombia blood	aerobic	1
<i>Enterobacteriaceae</i> (<i>E. coli</i> , <i>Klebsiella</i> etc.)	Drigalski	aerobic	1
Staphylococci (coagulase neg. + <i>S. aureus</i>)	Staphylococcus	aerobic	2
Enterococci (<i>E. faecalis</i> and <i>E. faecium</i>)	Enterococcosel	aerobic	2
Total anaerobes	Brucella blood	anaerobic	3
<i>Bacteroides</i>	Bile esculin	anaerobic	3
Bifidobacteria	Beerens	anaerobic	3
Clostridia	Brucella blood (alcohol treated sample)	anaerobic	3
<i>Clostridium difficile</i>	CCFA	anaerobic	3
Lactobacilli	Rogosa	anaerobic	3

Bacterial identification

Facultative bacteria

Enterobacteria were defined as Gram-negative rods growing aerobically on Drigalski agar plates. Isolates resembling enterobacteria were identified to the species level using the API20E biotyping system according to the manufacturer's instructions (API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France).

Staphylococci were identified by growth on staphylococcus agar, typical Gram stain appearance and a positive catalase reaction. Coagulase-positive staphylococci were identified as *S. aureus*, while other staphylococci were defined as coagulase-negative staphylococci (CoNS).

Enterococci were identified by growth on enterococcosel agar, esculin hydrolysis and typical Gram stain appearance. Yeasts were identified by growth on Sabouraud agar supplemented with penicillin and streptomycin, and typical Gram stain appearance.

Anaerobic bacteria

Isolates from plates incubated anaerobically were checked for inability to grow under aerobic conditions (Colombia blood agar plates, 37°C), and isolates unable to produce visible growth in 24 hours were defined as anaerobic bacteria. Weak growth under aerobic conditions was accepted for Gram-positive rods, as certain species of *Lactobacillus* and *Bifidobacterium* are able to grow aerobically.

Anaerobic Gram-negative rods growing on Bacteroides Bile Esculine agar were suspected to represent *Bacteroides* species. They were identified to the species level using the Rapid ID32A biotyping system (API systems), according to the manufacturer's instructions.

Isolates growing on Brucella blood agar inoculated with dilutions of alcohol-treated faeces and with a microscopical appearance of unbranched Gram-positive or Gram-variable rods were suspected to represent clostridial species. They were identified to the species level using the Rapid ID32A biotyping system (API systems). If a presumptive clostridial isolate died on subculture, it was still defined as belonging to the genus *Clostridium*, if spores were visible in the Gram-stained microscopic preparation. *Clostridium difficile* was identified by growth on CCFA, typical colony morphology, absence of lecithinase and lipase production and distinctive odour of p-cresol, Gram-stained appearance and Rapid ID32A.

Isolates growing on Beerens agar and resembling Gram-positive rods microscopically were subjected to PCR identifying bifidobacteria using the genus specific primers 5`-CCGGAATGCTCC-3` (PbiF1) and 5`-GACCATGCACCACCTGTGAA-3` (PbiR2) and the method described by Roy and Sirois (Roy and Sirois, 2000). Isolates obtained from Italian infants were sent frozen to the Department of Clinical Bacteriology, Göteborg, where they were analysed together with the Swedish isolates. Isolates from British infants were analysed at the Department of Medical Microbiology, St George's Hospital Medical School, London.

Unbranched Gram-positive rods isolated from Rogosa agar were subjected to PCR identifying bifidobacteria as described above, and isolates not reacting with the *Bifidobacterium* specific primers were assumed to be lactobacilli. These isolates were analyzed using multiplex PCR with primers specific for four different groups of lactobacilli as previously described (Song et al, 2000). All isolates that reacted with one of the group-specific primer pairs were defined as belonging to the genus *Lactobacillus*.

Microflora diversity – strain turn-over rate of key groups of intestinal bacteria

The strain turn-over rate in the microflora was determined for certain key bacterial groups, such as *E. coli*, *S. aureus* and lactobacilli. The PCR-based method RAPD (rapid amplification of polymorphic DNA) was used for strain identification. The principle of this method is that short primers of arbitrary sequence are allowed to bind to bacterial DNA under low-stringency conditions. PCR amplification results in a range of fragments with varying length. These fragments are separated by polyacrylamide electrophoresis and the pattern obtained after staining represents a unique "fingerprint" of the bacterial strain.

The RAPD methods used for *E. coli*, *S. aureus* and lactobacilli have been described in detail elsewhere (Lindberg et al., 2002; Nowrouzian et al., 2004, Ahrné et al., submitted manuscript).

RAPD analyses were applied on a subsample consisting of all strains of *E. coli*, *S. aureus* and lactobacilli from the intestinal microflora of 25 children per birth-cohort in order to assess differences in strain turn-over rates in different regions, as well as to assess the importance of microflora variability for allergy development.

Identification of bacterial species in faecal samples using T-RFLP and 16S rRNA gene sequencing

Certain bacterial species in the microflora are not culturable and culture conditions are bound to introduce a bias in the assessment of complex microbial populations. Molecular genetic methods for assessment of the normal intestinal microflora have been developed in the ALLERGYFLORA project.

The strategy employed for genetic analysis of the faecal microflora has been extraction of faecal DNA, followed by amplification of conserved regions of bacterial 16S rRNA genes (Pettersson et al, 2000), and analysis of the amplified fragments using Terminal-RFLP (T-RFLP).

In T-RFLP, PCR fragments are labeled due to use of fluorescence-labeled primers. The fragments are cut with restriction enzymes. Due to heterogeneity in the 16sRNA region, fragments of different lengths will be generated from different bacterial genera. These are separated according to size and detected by their fluorescence. The position of the fragment (i.e. the fluorescence peak) is indicative of the genus from which the DNA fragment that was amplified derived.

The method developed was described in detail in a recent publication (Wang et al. 2004). In brief, bacterial DNA from faecal samples was isolated and purified using the QIAmp DNA stool mini kit (Qiagen, Hilden, Germany). 16S rRNA genes were amplified in PCR using the primers ENV1 and ENV2, with ENV1 being fluorescently labeled at the 5' end with Cy5 (Amersham Biosciences, Uppsala, Sweden). PCR products from three assays were pooled and digested separately with each of the restriction enzymes MspI, RsaI, AluI and HaeIII. The fluorescently labeled terminal restriction fragments were separated and detected (ALFexpress II DNA sequencer with a ReproGel Long Read Gel). The sizes and peak areas were recorded and their relative abundance within a given T-RFLP pattern was calculated.

To identify which genera gave rise to peaks in different position, 16S rDNA clone libraries were constructed from the same faecal samples. Representative clones were subjected to T-RFLP and the T-RFLP patterns of the clones were compared with those of the corresponding faecal samples. When clones giving peaks found in the T-RFLP pattern of the faecal sample were identified, they were characterized by DNA sequencing.

Thus, by a combination of sequencing, RFLP and terminal-RFLP, an adequate picture of the dominant bacterial groups could be obtained without prior knowledge of their identity (Wang et al., 2004). In this way, we achieved one of the goals of the project – to develop a non-culture-dependent technique suitable to assess the composition of the infantile intestinal microflora. It should be stressed that the pattern obtained represent quantitatively dominant bacteria in the microflora only, while minor populations cannot be detected by this method.

Measurement of microflora heterogeneity using T-RFLP

To obtain a measure of the homogeneity/heterogeneity of the intestinal microflora as characterized by T-RFLP, the following method was applied:

The numbers of peaks were calculated for both *MspI* and *AluI* digests. For *MspI* peaks between 50 and 697 bp were included, while for *AluI* peaks between 30 to 697 bp were included.

The Shannon and Wiener diversity index (H') was calculated using the formula

$$H' = -\sum p_i \ln p_i$$

, where p_i is the percentage of the total peak area for each peak (Magguran, 1996; Krebs, 1999).

Clinical examination of signs and symptoms of allergy

At 18 months children were evaluated through a standard questionnaire with the following items: family demographic information, airway symptoms, symptoms from eyes or nose, eczema, gastro-intestinal symptoms, food related symptoms. The children underwent clinical assessment and a blood sample was analysed for total and specific IgE.

Clinical assessment of allergic diseases

Clinical examination included evaluation of weight and height, as well of signs of nasal obstruction, inspiratory and expiratory sounds, heavy breathing, signs of abdominal disease and eczema. Eczema was measured quantitatively using SCORAD. To this end, a new validated standardized software (Scorad-Card™) was used.

From this clinical examination, the child was diagnosed with asthma, allergic rhinoconjunctivitis, food allergy, or eczema according to standard criteria given below. For each diagnosis, the certainty of the diagnosis was graded by a scale from 1 to 4 (5 for food allergy), as defined in each table.

Criteria for clinical asthma

1.	Recurrent wheeze (at least 3 separate episodes) in association with common colds; no wheeze between the infections;
2.	Recurrent wheeze not only associated with common colds but also wheeze/symptoms in between infections, i.e. following physical activity, allergen exposure etc.
3.	Chronic/persistent wheeze with heavy breathing or cough for at least one month
4.	At least three episodes of wheeze in a child with other manifestations of allergy, i.e. food allergy or eczema.

Criteria for allergic rhinoconjunctivitis (ARC).

1	Symptoms from eyes and/or nose on pollen or animal exposure
2	As above plus positive RAST/SPT to pollens or animals

Criteria for clinical food allergy

1.	Immediate reaction after ingestion of the specific food, followed by a clear and prompt clinical improvement when eliminating the suspected food allergen
2.	Late-onset symptoms appearing several hours or days after ingestion of the suspected food, followed by a clear clinical response within 3 weeks when eliminating the suspected food allergen
3.	Food allergy based on symptoms and response to a food antigen as above, together with other signs of allergic disease
4.	Symptoms as above, but more than one organ system involved
5.	Symptoms as above supported by positive allergy tests, biopsies, or challenge tests.

Criteria for clinical (atopic) eczema

1	Typical spots with typical distribution
2	Itching rash with typical distribution which has come and gone during at least six months
3	Atopic eczema according to William's criteria.

IgE analysis

Blood (5 ml) was drawn by venipuncture and the serum was frozen at -70°C . Serum total IgE (IgE-FEIA, Pharmacia Diagnostics, Uppsala, Sweden) and specific IgE against common food allergens (egg, cows milk, codfish, peanut, soy bean) (ImmunoCAP-FEIA, Pharmacia Diagnostics) and inhalant allergens (Phadiatop, Pharmacia Diagnostics). The analysis was done in one laboratory, in order to minimize method variability.

For the purposes of statistical analyses, total IgE was dichotomised at 25kU/L. Only the presence of food-specific IgE has been included in this presentation, because of substantial variability in the prevalence of Phadiatop positivity between the centres (very probably due to the low prevalence of dust mite exposure and dust mite allergy in Göteborg).

Data analysis

Data was transferred from the Epi-Info databases to Stata (version 8) for data cleaning and statistical analysis. This report presents mainly genus-level microbiological information, to compare the intestinal colonization patterns of children with different life-style factors, such as siblings, pets, allergic heredity and antibiotic treatment of mother or child, and the relationship of intestinal colonization to allergic outcomes at 18 months. These results are presented systematically in the appendix.

For each genus, the presence or absence in the faeces at each time point (3 days, 1 week, 2 weeks, 4 weeks, 8 weeks, 6 months and 12 months) was analysed, for each centre separately, and all centres combined. The mean log₁₀ count for infants who were colonized was also analyzed as a continuous variable, for all centres combined. Finally, the cumulative incidence of colonization for each major bacterial group was derived by combining the data across the 7 time points (6 for anaerobic bacteria).

Tests of statistical significance were restricted to the cumulative colonization data, in order to limit the number of multiple comparisons, and to reduce the number of correlated measures for each child. The trend to earlier or later colonization in 2-group comparisons (eg. Caesarian v vaginal delivery, or eczema v no eczema) was tested for significance in a logistic regression framework, modeling the 2-group variable as the outcome, time-to-colonization as an ordinal 7- or 8-category variable (including never colonized), and centre as a 3-level categorical variable. This framework offer the flexibility to introduce additional factors, particularly when modeling the determinants of allergic outcomes.

3. Results

3.1 Method validity

A fundamental issue in the current project was method validity and generalizability. Only provided that the microbiological methods could be reliably standardized and harmonized could direct comparisons be made across the centres. No previous efforts have been made, to the best of our knowledge, to directly compare colonization patterns between different geographical regions using the same methodology.

Quantitative culture of faecal samples requires standardized and adequate sample transportation and handling. Many anaerobic bacteria in faecal samples die in contact with ambient oxygen. Faecal samples that have been frozen do not yield the same results as direct culture of fresh samples. The methodology worked out consisted of collection and transport of faecal samples in an air-tight bag with anaerobic atmosphere and culture within 24 hours. The sample bag was kept refrigerated in the homes until being delivered to the laboratory.

Bacterial recovery and population counts after storage of faecal samples

The effect of storage of faecal specimens in the sample bag in an anaerobic atmosphere was examined. As seen in Table 2, storage for up to 40 - 50 hours in the refrigerator had little effect on recovery and population counts of major groups of faecal bacteria. The counts of facultative bacteria were practically identical in freshly cultured and stored samples. In one sample, coagulase-negative staphylococci (CoNS) were not recovered after storage. However, only a single CoNS colony was found in the freshly cultured sample (detection limit: $10^{2.52}$ CFU/g faeces).

The total counts of anaerobes were also mostly unaffected by storage under anaerobic conditions (Table 2). The lower mean counts of clostridia after prolonged storage was due to discrepant result for one sample. The isolate did not survive subculture and could thus not be speciated by biotyping. When isolated from the freshly cultured sample, spores were observed in the Gram-stained preparation. The isolate was thus defined as *Clostridium* spp according to the established criteria (see Materials and Methods). When isolated from the same sample stored for 40 hours, no spores were observed in the Gram-stained preparation. The isolate could, thus, not be defined as a *Clostridium* species. Since this isolate was found in quite high numbers, the total clostridial counts were affected.

Another four faecal samples were used to test the effect of storage for 70-120 h. The facultatives were still little affected, as were the counts of total anaerobes (Table 3). However, the counts of anaerobes obtained on selective media were in some cases reduced. For example, in one sample *Bacteroides* were isolated in high counts ($10^{10.5}$ CFU/g faeces) on immediate culture, but were not recovered when the sample was cultured after 70 hours.

In contrast, bifidobacteria were isolated in a stored sample while not identified in the freshly cultured portion of the sample. This was because the bacteria in this preliminary analysis were only tentatively identified by Gram-stained appearance. Thus, only bacteria that appeared as branched or club-shaped rods in the Gram-stained preparation were identified as bifidobacteria. In the freshly cultured sample, the same bacteria appeared as unbranched straight rods in the Gram-stained preparation and

were therefore not defined as bifidobacteria in this preliminary analysis. However, they would not have been missed in the final analysis, when all isolates from Beerens agar are identified using a *Bifidobacterium*-specific PCR.

In summary, *Bacteroides* appeared to be more sensitive to prolonged storage than other anaerobic bacteria. This may have bearing on the results obtained in the inter-laboratory comparisons, which were based on simultaneous analysis of a fresh faecal sample which was divided in three portions and sent to one or two of the laboratories by air. In some cases, *Bacteroides* present in the faecal samples may have died during shipping. For the processing of infantile faecal samples in the local laboratories, the methodology appeared to be well suited.

Table 2. Bacterial/microbial recovery after 40 - 50 hours storage.

Microbes	fresh samples		stored samples	
	n/n	log counts (mean)	n/n	log counts (mean)
Total facultatives	4/4	8.6	4/4	8.6
<i>Enterobacteriaceae</i>	4/4	7.8	4/4	7.8
<i>E. coli</i>	3/4	8.7	3/4	8.8
Other	3/4	6.8	3/4	6.7
Enterococci	4/4	7.8	4/4	7.8
Staphylococci	4/4	5.6	3/4	5.4
<i>S. aureus</i>	2/4	6.1	2/4	5.9
CoNS	4/4	4.5	3/4	4.4
Yeasts	3/4	4.2	3/4	4.2
Total anaerobes	4/4	10.3	4/4	10.3
<i>Bacteroides</i> *	4/4	9.2	4/4	9.0
Bifidobacteria**	3/4	8.7	3/4	9.0
Clostridia	4/4	7.2	4/4	6.2
<i>C. difficile</i>	0/4	-	0/4	-

Table 3. Bacterial/microbial recovery and population counts after 70 - 120 hours storage.

Microbes	fresh samples		stored samples	
	n/n	log counts (mean)	n/n	log10 counts (mean)
Total aerobes	4/4	8.5	4/4	8.3
<i>Enterobacteriaceae</i>	4/4	7.6	4/4	7.4
<i>E. coli</i>	4/4	7.5	4/4	7.3
other	4/4	6.3	4/4	6.1
Enterococci	4/4	7.6	4/4	7.4
Staphylococci	4/4	4.5	4/4	4.5
<i>S. aureus</i>	0/4	-	0/4	-
CoNS	4/4	4.5	4/4	4.5
Yeasts	3/4	3.7	3/4	4.0
Total anaerobes	4/4	10.4	4/4	10.2
<i>Bacteroides</i> *	3/4	10.1	2/4	9.4
Bifidobacteria**	3/4	10.4	4/4	9.7
Clostridia	4/4	6.3	4/4	5.2
<i>C. difficile</i>	0/4	-	0/4	-

The log10 counts were calculated for samples positive for the bacterium at both culture occasions.

*Three isolates were defined as *Bacteroides* based on growth on BBE agar, colony morphology and Gram-stain appearance only, as the isolates died on subculture.

**Presumptive identification. Isolates of Gram-positive rods with bifid or club-shaped ends growing on Beerens agar were defined as bifidobacteria.

Intra-laboratory variability in bacterial recovery and population counts in faecal samples

The intra-laboratory variation was assessed by dividing two faecal specimens into each three portions, which were cultured simultaneously by three technicians in the Swedish laboratory. The results are presented in Table 4. The total numbers of facultatives varied up to 0.5 log units, while the total anaerobic counts varied very little. Among the facultatives, *Enterobacteriaceae* showed the lowest variability and staphylococci the highest. The variations in clostridial recovery rate and counts were related to the fact that in some cases the isolates could not be subcultured, which was a prerequisite for identification by Rapid ID 32A. If spores were seen in the Gram-stained preparation from the original culture, the colonies were nevertheless defined as clostridia, even if they could not be speciated. Organisms appearing as straight rods, but with no spores in the preparation, were not classified as clostridia.

Table 4. Bacterial/microbial recovery and population counts in two faecal samples cultured simultaneously by three different technicians in the Swedish laboratory.

Microbes	Technician 1		Technician 2		Technician 3	
	isolation rate	log CFU (mean)	isolation rate	log CFU (mean)	isolation rate	log CFU (mean)
Total facultatives	2/2	7.7	2/2	7.6	2/2	7.2
<i>Enterobacteriaceae</i>	2/2	5.8	2/2	5.9	2/2	5.6
<i>E. coli</i>	0/2	-	0/2	-	0/2	-
Other	2/2	5.8	2/2	5.9	2/2	5.6
Enterococci	2/2	6.8	2/2	7.3	2/2	7.5
Staphylococci	2/2	5.4	2/2	4.6	2/2	5.3
<i>S. aureus</i>	0/2	-	0/2	-	0/2	-
CoNS	2/2	5.4	2/2	4.6	2/2	5.3
Yeasts	2/2	4.0	2/2	3.9	2/2	3.4
Total anaerobes	2/2	10.5	2/2	10.3	2/2	10.4
<i>Bacteroides</i>	2/2	9.4	2/2	8.8	2/2	9.7
Bifidobacteria*	2/2	9.3	2/2	9.1	2/2	9.1
Clostridia	2/2	7.8	1/2	5.6	2/2	6.7
<i>C. difficile</i>	0/2	-	0/2	-	0/2	-

The log₁₀ counts were calculated for samples positive for the bacterium at both culture occasions.

*Presumptive identification. Isolates of Gram-positive rods with bifid or club-shaped ends growing on Beerens agar were defined as bifidobacteria.

3.2. Method compatibility between centres

Inter-laboratory variability in bacterial recovery and population counts in faecal samples

The inter-laboratory variation was monitored by sending out freshly collected faecal samples to one or both partner laboratories at regular intervals. The samples were cultured and analysed in parallel.

Table 5 shows the results for 10 samples analysed in parallel at the Department of Clinical Bacteriology, Göteborg and the Medical Microbiology laboratory, Sandro Pertinini Hospital, Rome. For most bacterial groups, there was very good agreement between the two laboratories. However, some differences were observed. In one sample, enterobacteria other than *E. coli* were recovered in the Swedish, but not the Italian laboratory. These bacteria were isolated from the Drigalski agar plate in two log lower counts than *E. coli*, and were most likely missed in the Italian laboratory due to overgrowth by *E. coli*. In the sample in which CoNS were isolated in Sweden but not Italy, *S. aureus* were present in 1.5 log units higher counts on the plates. Such differences will always be encountered when quantitative cultures are performed on complex bacterial mixtures, since minor differences in the number of free lying colonies on appropriate dilutions may result in the recovery or non-recovery of subdominant bacterial groups. In the one sample yielding yeasts in the Swedish but not the Italian laboratory, a single yeast colony was present on the lowest dilution on Saboraud agar, which gave a log count of $10^{2.52}$ CFU/g faeces.

Table 6 shows the results for 12 samples analysed in parallel at the Department of Clinical Bacteriology, Göteborg and the Department of Medical Microbiology, St George's Hospital Medical School, London. Also in this comparison, there was very good agreement for most bacterial groups between the two laboratories. In one sample, enterobacteria other than *E. coli* (i.e. *Proteus vulgaris*), were recovered from the Drigalski plates in the Swedish, but not the British laboratory where they were overgrown by *E. coli*. In another sample, staphylococci (CoNS) were isolated in London but not in Göteborg. In this case, only four CoNS colonies were present on the lowest dilution on staphylococcus agar ($10^{3.12}$ CFU/g faeces) in London, and stochastic variation can easily explain why these did not appear when cultured in the Swedish laboratory. Along the same lines, 7/10 samples yielded yeasts in the Swedish laboratory, but only 4/10 that were cultured in London. In the three cases where yeasts were isolated in Göteborg but not in London, only 1, 2 and 4 yeast colonies, respectively, were present on the lowest dilution on Saboraud agar in the Swedish laboratory. In samples that contained more substantial quantities of yeasts, there was little variation in recovery rate between the two laboratories.

Bacteroides were isolated from 9 of 12 samples in Göteborg, and from 6 of 12 samples in London. When present, *Bacteroides* counts were above $10^{7.5}$ CFU/g faeces. The lower recovery rate in the British laboratory could be due to selective loss of *Bacteroides* during transport of the samples from Sweden to England. However, this was observed to a much lower degree with samples transported from Sweden to Italy. We cannot exclude that the lower recovery rate in London reflects unidentified differences in handling and culturing of samples between the British and the Swedish and Italian laboratories.

All but one sample yielded bacteria on Beerens agar presumptively identified as bifidobacteria in both Sweden and England. In the one sample yielding these bacteria in the Swedish lab only, non-

bifid Gram-positive rods were present on the Beerens plates in London in similar concentrations as the bifid Gram-positive rods isolated in the Swedish laboratory, most likely representing the same bacteria. As all Gram-positive rods from Beerens agar in the ALLERGYFLORA study are identified by *Bifidobacterium*-specific PCR, the end results will not be affected.

Table 5. Bacterial/microbial recovery and population counts in 10 faecal samples cultured simultaneously at the laboratories in Göteborg and Rome.

Microbes	Göteborg		Rome	
	n/n	log CFU (mean)	n/n	log CFU (mean)
Total facultatives	9/9	8.7	9/9	8.4
<i>Enterobacteriaceae</i>	9/9	8.2	9/9	7.6
<i>E. coli</i>	8/9	8.6	8/9	7.9
Other	7/9	6.9	6/9	6.2
Enterococci	9/9	7.7	9/9	7.1
Staphylococci	8/9	5.0	8/9	4.9
<i>S. aureus</i>	4/9	5.4	4/9	4.9
CoNS	7/9	4.6	6/9	4.6
Yeasts	7/9	3.5	6/9	3.0
Total anaerobes	10/10	10.2	10/10	9.7
<i>Bacteroides</i> *	8/10	9.4	8/10	8.9
Bifidobacteria**	10/10	9.0	10/10	8.7
Clostridia	10/10	6.1	10/10	6.0
<i>C. difficile</i>	2/10	5.3	2/10	6.1

The log₁₀ counts were calculated for samples positive for the bacterium in both laboratories. Analyses of aerobic bacteria and yeasts were performed on 9 of 10 samples, as for one sample the culture plates incubated aerobically were lost for analyses in the Swedish laboratory.

*Two isolates were defined as *Bacteroides* based on growth on BBE agar, colony morphology and Gram-stain appearance only, as the isolates died on subculture.

**Presumptive identification. Isolates of Gram-positive rods with bifid or club-shaped ends growing on Beerens agar were defined as bifidobacteria.

Table 6. Bacterial/microbial recovery and population counts in 12 faecal samples cultured simultaneously at the laboratories in Göteborg and London.

Microbes	Göteborg		London	
	isolation rate	logCFU (mean)	isolation rate	logCFU (mean)
Total facultatives	11/11	8.6	11/11	8.4
<i>Enterobacteriaceae</i>	11/11	7.9	11/11	7.8
<i>E. coli</i>	10/11	8.2	10/11	8.1
Other	8/11	6.5	7/11	6.4
Enterococci	11/11	7.5	11/11	7.2
Staphylococci	10/11	5.0	11/11	4.8
<i>S. aureus</i>	5/11	5.3	5/11	4.7
CoNS	9/11	4.6	10/11	4.5
Yeasts	7/10	4.4	4/10	4.3
Total anaerobes	12/12	10.1	12/12	10.1
<i>Bacteroides</i> *	9/12	9.1	6/12	8.5
Bifidobacteria**	12/12	9.1	11/12	8.9
Clostridia	12/12	5.7	12/12	6.2
<i>C. difficile</i> *	2/12	5.3	2/12	5.2

The log10 counts were calculated for samples positive for the bacterium in both laboratories. Analyses of aerobic bacteria and yeasts were performed on 11 of 12 samples, as for one sample the culture plates incubated aerobically were lost for analyses in the Swedish laboratory.

*Five isolates were defined as *Bacteroides* based on growth on BBE agar, colony morphology and Gram-stain appearance only, as the isolates died on subculture. Two isolates were defined as *C. difficile* based solely on growth on CCFA, colony morphology, p-cresol odour, lecithinase and lipase negative, and Gram-stain appearance.

**Presumptive identification. Isolates of Gram-positive rods with bifid or club-shaped ends growing on Beerens agar were defined as bifidobacteria.

3.3 Clinical outcome

Prevalence of different background variables in the three centres

Some “traditional” risk factors for allergic disease showed variance between the three centres (Table 7). Parental allergy was very common in the Swedish cohort (68% of mothers and 61% of fathers were atopic) as well as in the British cohort (where 51% of mothers and 48% of fathers were atopic), but less common in the Italian cohort (where 29% and 37% of mothers and fathers were atopic).

The rate of caesarean section was high in Rome (39%) and London (30%), but moderate in Göteborg (15%). Administration of antibiotics to the mother during pregnancy showed the same pattern, being most frequent in Rome (where 51% of all pregnant women were given antibiotics), followed by London (36%) and Göteborg (6%). Prescription of antibiotics to the child was common in London (36% were given antibiotics during the first six months of life) and Rome (29%), but relatively uncommon in Göteborg (8%).

The presence of siblings and pets in the family was quite uniform across the three centres. London had the lowest rate of continued breast-feeding past six months and the highest rate of non-parental day care by six months of age (Table 7).

Prevalence of different manifestations of allergy in the three centres

Table 8 shows the prevalence of different manifestations of allergy in the different centres and in the combined cohort. Grade 3 eczema affected approximately 30% of Swedish and British infants by 18 months of age, but only 6% of Italian infants.

Clinical asthma was not seen among the Italian infants and was not very common either in London (4%) and Göteborg (5%). This was expected, due to the young age of the children when examined. Allergic rhinitis was even less common, which is also characteristic of the age group. Only a single child in the Swedish cohort had confirmed (grade 3) rhinitis (Table 8).

Proven (grade 5) food allergy was rare, being documented only in two British infants. However, approximately 20% of the infants across the centres had experienced immediate or late-onset symptoms in relation to consumption of a certain food item, symptoms which were eliminated when the food was excluded from the diet (Table 8).

Elevated (>25 U) IgE was very common. Almost half of the Italian infants and a third of the Swedish and British infants had elevated IgE levels (“elevated” defined as > 1 SD above the mean for the age group). Specific IgE to food mix was present in 20-25 of the infants across the three centres (Table 8).

To summarize, clinical eczema was by far the most common manifestation of allergy in the age group. This manifestation was uncommon in Italian cohort, while one third of the infants in the Swedish and British cohorts had clinical eczema according to William’s criteria by 18 months of age.

Table 7. Prevalence of different background variables in the three cohorts.

Risk Factors	Centre				
		Goteborg n (col%)	London n (col%)	Rome n (col%)	All centres n (col%)
Mode of delivery	Caesarean	17(14.66)	32(29.63)	39(39.00)	88(27.16)
	Vaginal	99(85.34)	76(70.37)	61(61.00)	236(72.84)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Antibiotics to mother in pregnancy	No	109(93.97)	69(63.89)	49(49.00)	227(70.06)
	Yes	7(6.03)	39(36.11)	51(51.00)	97(29.94)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Presence of siblings	No	63(54.31)	57(52.78)	54(54.00)	174(53.70)
	Yes	53(45.69)	51(47.22)	46(46.00)	150(46.30)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Antibiotics to child by 6 months	No	107(92.24)	69(63.89)	71(71.00)	247(76.23)
	Yes	9(7.76)	39(36.11)	29(29.00)	77(23.77)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Pet ownership at 6 months	No	99(85.34)	88(81.48)	75(75.00)	262(80.86)
	Yes	17(14.66)	20(18.52)	25(25.00)	62(19.14)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
(Non-parental) child care at 6 months	No	110(94.83)	84(77.78)	85(85.00)	279(86.11)
	Yes	6(5.17)	24(22.22)	15(15.00)	45(13.89)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Exclusively breastfed at 6 months	No	87(76.32)	95(87.96)	63(63.00)	245(76.09)
	Yes	27(23.68)	13(12.04)	37(37.00)	77(23.91)
	Total	114(100.00)	108(100.00)	100(100.00)	322(100.00)
	Missing	2	0	0	2
Maternal history of allergy	No	37(31.90)	53(49.07)	71(71.00)	161(49.69)
	Yes	79(68.10)	55(50.93)	29(29.00)	163(50.31)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)
Paternal history of allergy	No	45(38.79)	56(51.85)	63(63.00)	164(50.62)
	Yes	71(61.21)	52(48.15)	37(37.00)	160(49.38)
	Total	116(100.00)	108(100.00)	100(100.00)	324(100.00)

Table 8. Prevalence of different manifestations of allergy by 18 months of age

Clinical Outcomes	Centre				
		Goteborg n (col%)	London n (col%)	Rome n (col%)	All centres n (col%)
Clinical eczema	0	73(62.93)	53(50.00)	67(84.81)	193(64.12)
	1	7(6.03)	5(4.72)	2(2.53)	14(4.65)
	2	2(1.72)	13(12.26)	5(6.33)	20(6.64)
	3	34(29.31)	35(33.02)	5(6.33)	74(24.58)
	Total	116(100.00)	106(100.00)	79(100.00)	301(100.00)
	Missing	0	2	21	23
Clinical eczema at 18 months (binary)	No	82(70.69)	71(66.98)	74(93.67)	227(75.42)
	Yes	34(29.31)	35(33.02)	5(6.33)	74(24.58)
	Total	116(100.00)	106(100.00)	79(100.00)	301(100.00)
	Missing	0	2	21	23
Clinical asthma by 18 months	0	103(89.57)	73(67.59)	66(83.54)	242(80.13)
	1	2(1.74)	23(21.30)	9(11.39)	34(11.26)
	2	4(3.48)	8(7.41)	4(5.06)	16(5.30)
	3	6(5.22)	4(3.70)	0(0.00)	10(3.31)
	Total	115(100.00)	108(100.00)	79(100.00)	302(100.00)
	Missing	1	0	21	22
Clinical rhinitis at 18 months	0	109(95.61)	87(8.56)	72(91.14)	268(89.04)
	1	2(1.75)	20(18.52)	6(7.59)	28(9.30)
	2	2(1.75)	1(0.93)	1(1.27)	4(1.33)
	3	1(0.88)	0(0.00)	0(0.00)	1(0.33)
	Total	(100.00)	(100.00)	(100.00)	(100.00)
	Missing	2	0	21	22
Clinical food allergy	0	82(78.85)	79(79.80)	47(74.60)	208(78.20)
	1	16(15.38)	8(8.08)	8(12.70)	32(12.03)
	2	5(4.81)	8(8.08)	6(9.52)	19(7.14)
	3	1(0.96)	2(2.02)	2(3.17)	5(1.88)
	5	0(0.00)	2(2.02)	0(0.00)	2(0.75)
	Total	(100.00)	(100.00)	(100.00)	(100.00)
	Missing	12	9	37	58
Specific IgE to food mix at 18 months	No	82(78.85)	79(79.80)	47(74.60)	208(78.20)
	Yes	22(21.15)	20(20.20)	16(25.40)	58(21.80)
	Total	104(100.00)	99(100.00)	63(100.00)	266(100.00)
	Missing	12	9	37	58
Total serum IgE at 18 months	< 25	79(71.82)	66(66.67)	33(52.38)	178(65.44)
	≥ 25	31(28.18)	33(33.33)	30(47.62)	94(34.56)
	Total	110(100.00)	99(100.00)	63(100.00)	272(100.00)
	Missing	6	9	37	52

Risk of developing eczema by 18 months of age in relation to life-style factors

The risk factors for development of clinical eczema by 18 months of age is shown in Table 9.

Clinical eczema by 18 months of age was strongly positively associated with maternal history of allergy (OR 2.2, $p=0.005$, Fisher's exact test). Clinical eczema was also related positively to specific IgE responses to the food-mix (OR 3.2, $p=0.003$), but totally unrelated to elevated levels of total IgE (OR 1.1, $p=0.77$).

A moderate positive association was seen between clinical eczema and administration of antibiotics to the child in the first six months of life. Vaginally-delivered infants were slightly more likely to develop eczema than caesarean section-delivered infants, which may be a consequence of the high rate of section-delivery in the Italian cohort, which had low prevalence of eczema. The same pattern was seen with exclusive breast-feeding at six months of age which was weakly negatively related to eczema, a relation which could depend on the high rate of continued breast-feeding in the Italian cohort.

Risk of developing increased levels of specific IgE to food-mix in relation to life-style factors

Table 10 shows the risk of developing increased levels of specific IgE to food-mix at 18 months of age, in relation to life-style factors in the three different cohorts.

Specific IgE to food-mix was strongly positively correlated with increased levels of total IgE (OR 8.2, $p<0.0001$) and to clinical eczema (OR 3.2, $p=0.003$). It was weakly connected to maternal history of allergy and to non-parental day care at six months of age.

Risk of developing increased levels of total IgE in relation to life-style factors

Table 11 shows the risk of developing increased levels of total IgE at 18 months of age, in relation to life-style factors in the three different cohorts.

Increased levels of total IgE were positively related to pet ownership (OR 1.9, $p=0.04$). Increased total IgE was negatively associated with paternal allergy (OR 0.6, $p=0.04$) and unrelated to clinical eczema (OR 1.1, $p=0.77$).

Table 9. Risk of developing eczema in relation to life-style factors.

Risk Factors	Clinical eczema at 18 months				
		No n (row%)	Yes n (row%)	Total n (row%)	Missing
Mode of delivery	Caesarean	67(80.72)	16(19.28)	83(100.00)	5
	Vaginal	160(73.39)	58(26.61)	218(100.00)	18
	Total	227(75.42)	74(24.58)	301(100.00)	23
Antibiotics to mother in pregnancy	No	154(73.33)	56(26.67)	210(100.00)	17
	Yes	73(80.22)	18(19.78)	91(100.00)	6
	Total	227(75.42)	74(24.58)	301(100.00)	23
Presence of siblings	No	126(77.30)	37(22.70)	163(100.00)	11
	Yes	101(73.19)	37(26.81)	138(100.00)	12
	Total	227(75.42)	74(24.58)	301(100.00)	23
Antibiotics to child by 6 months	No	184(79.31)	48(20.69)	232(100.00)	15
	Yes	43(62.32)	26(37.68)	69(100.00)	8
	Total	227(75.42)	74(24.58)	301(100.00)	23
Pet ownership at 6 months	No	181(74.79)	61(25.21)	242(100.00)	20
	Yes	46(77.97)	13(22.03)	59(100.00)	3
	Total	227(75.42)	74(24.58)	301(100.00)	23
(Non-parental) child care at 6 months	No	199(76.25)	62(23.75)	261(100.00)	18
	Yes	28(70.00)	12(30.00)	40(100.00)	5
	Total	227(75.42)	74(24.58)	301(100.00)	23
Exclusively breastfed at 6 months	No	167(73.25)	61(26.75)	228(100.00)	19
	Yes	60(82.19)	13(17.81)	73(100.00)	4
	Total	227(75.42)	74(24.58)	301(100.00)	23
Maternal history of allergy	No	120(82.76)	25(17.24)	145(100.00)	16
	Yes	107(68.59)	49(31.41)	156(100.00)	7
	Total	227(75.42)	74(24.58)	301(100.00)	23
Paternal history of allergy	No	113(76.35)	35(23.65)	148(100.00)	16
	Yes	114(74.51)	39(25.49)	153(100.00)	7
	Total	227(75.42)	74(24.58)	301(100.00)	23
Clinical eczema at 18 months	No	227(100.00)	0(0.00)	227(100.00)	0
	Yes	0(0.00)	74(100.00)	74(100.00)	0
	Total	227(100.00)	74(100.00)	301(100.00)	23
	Missing	0	0		23
Specific IgE to food mix at 18 months	No	164(79.61)	42(20.39)	206(100.00)	2
	Yes	32(55.17)	26(44.83)	58(100.00)	0
	Total	196(74.24)	68(25.76)	264(100.00)	60
	Missing	31	6		21
Total serum IgE at 18 months	< 25	131(74.43)	45(25.57)	176(100.00)	2
	≥ 25	68(72.34)	26(27.66)	94(100.00)	0
	Total	199(73.70)	71(26.30)	270(100.00)	54
	Missing	28	3		21

Table 10. Risk of developing increased levels of food-specific IgE in relation to life-style factors

Risk Factors	Specific IgE to food mix at 18 months				
		No n (row%)	Yes n (row%)	Total n (row%)	Missing
Mode of delivery	Caesarean	57(80.28)	14 (19.72)	71 (100.00)	17
	Vaginal	151(77.44)	44 (22.56)	195 (100.00)	41
	Total	208(78.20)	58 (21.80)	266 (100.00)	58
Antibiotics to mother in pregnancy	No	150 (78.95)	40 (21.05)	190(100.00)	37
	Yes	58 (76.32)	18 (23.68)	76(100.00)	21
	Total	208(78.20)	58 (21.80)	266 (100.00)	58
Presence of siblings	No	113 (77.93)	32 (22.07)	145(100.00)	29
	Yes	95 (78.51)	26 (21.49)	121(100.00)	29
	Total	208 (64.20)	58 (21.80)	266(100.00)	58
Antibiotics to child by 6 months	No	160 (78.05)	45 (21.95)	205(100.00)	42
	Yes	48 (78.69)	13 (21.31)	61(100.00)	16
	Total	208(78.20)	58 (21.80)	266(100.00)	58
Pet ownership at 6 months	No	169 (78.60)	46 (21.40)	215(100.00)	47
	Yes	39 (76.47)	12 (23.53)	51(100.00)	11
	Total	208(78.20)	58 (21.80)	266(100.00)	58
(Non-parental) child care at 6 months	No	187 (79.57)	48 (20.43)	235(100.00)	44
	Yes	21 (67.74)	10 (32.26)	31(100.00)	14
	Total	208(78.20)	58 (21.80)	266(100.00)	58
Exclusively breastfed at 6 months	No	153 (77.66)	44 (22.34)	197(100.00)	50
	Yes	55 (79.71)	14 (20.29)	69(100.00)	8
	Total	208(78.20)	58 (21.80)	266(100.00)	58
Maternal history of allergy	No	102 (83.61)	20 (16.39)	122(100.00)	39
	Yes	106 (73.61)	38 (26.39)	144(100.00)	19
	Total	208(78.20)	58 (21.80)	266(100.00)	58
Paternal history of allergy	No	96 (75.59)	31 (24.41)	127(100.00)	37
	Yes	112 (80.58)	27 (19.42)	139(100.00)	21
	Total	208(78.20)	58 (21.80)	266(100.00)	58
Clinical eczema at 18 months	No	164 (83.67)	32 (16.33)	196(100.00)	31
	Yes	42 (61.76)	26 (38.24)	68(100.00)	6
	Total	206(78.03)	58 (21.97)	264(100.00)	60
	Missing	2	0	2	21
Specific IgE to food mix at 18 months	No	208(100.00)	0(0.00)	208(100.00)	0
	Yes	0(0.00)	58 (100.00)	58 (100.00)	0
	Total	208(78.20)	58 (21.80)	266(100.00)	58
	Missing	0	0	0	58
Total serum IgE at 18 months	< 25	156 (91.23)	15 (8.77)	171(100.00)	7
	≥ 25	52 (55.91)	41 (44.09)	93(100.00)	1
	Total	208(78.79)	56 (21.21)	264(100.00)	60
	Missing	0	2	2	50

Table 11. Risk of developing increased levels of total IgE in relation to life-style factors

Risk Factors	Total serum IgE \geq 25 kU/L at 18 months				
		No n (row%)	Yes n (row%)	Total n (row%)	Missing
Mode of delivery	Caesarean	48(66.67)	24(33.33)	72(100.00)	16
	Vaginal	130(65.00)	70(35.00)	200(100.00)	36
	Total	178(65.44)	94(34.56)	272(100.00)	52
Antibiotics to mother in pregnancy	No	129(65.48)	68(34.52)	197(100.00)	30
	Yes	49(65.33)	26(34.67)	75(100.00)	22
	Total	178(65.44)	94(34.56)	272(100.00)	52
Presence of siblings	No	99(66.89)	49(33.11)	148(100.00)	26
	Yes	79(63.71)	45(36.29)	124(100.00)	26
	Total	178(65.44)	94(34.56)	272(100.00)	52
Antibiotics to child by 6 months	No	142(67.30)	69(32.70)	211(100.00)	36
	Yes	36(59.02)	25(40.98)	61(100.00)	16
	Total	178(65.44)	94(34.56)	272(100.00)	52
Pet ownership at 6 months	No	150(68.49)	69(31.51)	219(100.00)	43
	Yes	28(52.83)	25(47.17)	53(100.00)	9
	Total	178(65.44)	94(34.56)	272(100.00)	52
(Non-parental) child care at 6 months	No	158(65.56)	83(34.44)	241(100.00)	38
	Yes	20(64.52)	11(35.48)	31(100.00)	14
	Total	178(65.44)	94(34.56)	272(100.00)	52
Exclusively breastfed at 6 months	No	139(68.14)	65(31.86)	204(100.00)	43
	Yes	39(57.35)	29(42.65)	68(100.00)	9
	Total	178(65.44)	94(34.56)	272(100.00)	52
Maternal history of allergy	No	82(65.20)	43(34.40)	125(100.00)	36
	Yes	96(65.31)	51(34.69)	147(100.00)	16
	Total	178(65.44)	94(34.56)	272(100.00)	52
Paternal history of allergy	No	77(59.23)	53(40.77)	130(100.00)	34
	Yes	101(71.13)	41(28.87)	142(100.00)	18
	Total	178(65.44)	94(34.56)	272(100.00)	52
Clinical eczema at 18 months	No	131(65.83)	68(34.17)	199(100.00)	28
	Yes	45(63.38)	26(36.62)	71(100.00)	3
	Total	176(65.19)	94(34.81)	270(100.00)	54
	Missing	2	0		21
Specific IgE to food mix at 18 months	No	156(75.00)	52(25.00)	208(100.00)	0
	Yes	15(26.79)	41(73.21)	56(100.00)	2
	Total	171(64.77)	93(35.23)	264(100.00)	60
	Missing	7	1		50
Total serum IgE at 18 months	< 25	178(100.00)	0(0.00)	178(100.00)	0
	\geq 25	0(0.00)	94(100.00)	94 (100.00)	0
	Total	178(100.00)	94(100.00)	272(100.00)	52
	Missing	0	0		52

3.4 Intestinal colonization pattern - comparison between centres and effect of life-style factors

Colonization by each of the major bacterial groups is shown in the Appendix. The bacterial groups are consecutively numbered as follows: 1) *Enterobacteriaceae*, 2) *E. coli*, 3) *Klebsiella*, 4) Other *Enterobacteriaceae* than *E. coli* or *Klebsiella*, 5) *Staphylococcus aureus*, 6) Coagulase-negative staphylococci, 7) Enterococci, 8), *Bacteroides*, 9), Bifidobacteria, 10) Lactobacilli, 11) Clostridia.

For each bacterial group, the results are showed as colonization rates by age in each of the three centres as well as in the combined cohort. A cumulative colonization rate is also calculated for the combined cohort. Lastly, the stool population levels of the bacterial group in question in infants colonized by these bacteria is shown (data being shown for the combined cohort).

The influence of the following life-style factors on colonization rates (measured as indicated above) has been calculated and is shown on a separate data and figure sheet for each bacterium and life-style factor: 1) Mode of delivery (caesarean section versus vaginal delivery), 2) Administration of antibiotics to mother during pregnancy (yes/no), 3) Presence of siblings in household (yes/no), 4) Administration of antibiotics to child during first six months of life (yes/no), 5) Presence of pets in household (yes/no), 6) Attendance at day care by six months of age (yes/no), 7) Feeding mode (exclusive breast-feeding by six months of age: yes/no), 8) Maternal history allergy (yes/no), and 9) Paternal history of allergy.

Enterobacteriaceae

Colonization by *Enterobacteriaceae* over the first year of life in the three different cohorts, in relation to life-style factors are shown in Figures 1.1-1.9 in the Appendix.

In all centres, colonization by *Enterobacteriaceae* was markedly more rapid in infants born vaginally than by caesarean section. In general, colonization by *Enterobacteriaceae* occurred more slowly in the Swedish cohort than in the Italian and British cohorts. Furthermore, in the Swedish cohort, *Enterobacteriaceae* colonization was delayed in section-delivered as compared to vaginally-delivered infants up to six months of age. *Section*-delivered infants in Rome and London caught up with respect to enterobacterial colonization already by one month of age. This suggests that the environment of the Swedish birth-cohort provides a poorer source of enterobacteria than the environments of the two other cohorts.

Children with siblings were somewhat more rapidly colonized by *Enterobacteriaceae* than those without siblings, but this could be an effect of caesarean section being more frequent in first-born infants. There was a weak tendency of more rapid colonization in children whose parents kept pets.

In general, enterobacterial colonization was unaffected by antibiotic treatment of the child, which was due to opposite effects in the different centres – increased enterobacterial colonization in antibiotic-treated Swedish children and decreased enterobacterial colonization in antibiotic-treated British and Italian children, compared to their non-treated counterparts.

Children of non-allergic mothers were slightly earlier colonized by enterobacteria than children of mothers with a history of allergy.

***Escherichia* spp (foremost *E. coli*)**

Colonization by *Escherichia* spp over the first year of life in the three different cohorts, in relation to life-style factors are shown in Figures 2.1-2.9 in the Appendix. *E. coli* was the *Escherichia* species that was totally dominant. *E. coli* is used instead of *Escherichia* hereafter.

Colonization by *E. coli* was dramatically delayed in infants delivered by caesarean section. The difference between vaginal and section-delivered infants was evident up to six months of age in all centres.

Colonization by *E. coli* occurred slightly earlier in children with siblings than in single children. There was no effect of pets in the household and no effect by antibiotics given to the child. In all three cohorts, children who were breast-fed exclusively for six months or more were earlier colonized by *E. coli* compared to children weaned earlier.

Klebsiella

Infants delivered by caesarean section were more often colonized by *Klebsiella* during the first six months of life in all three centres. Children who were exclusively breast-fed for at least six months were less often colonized by *Klebsiella* compared with weaned infants in all three centres.

Other enterobacteria

Enterobacteriaceae other than *E. coli* and *Klebsiella*, e.g. *Enterobacter*, *Citrobacter*, *Proteus*, and *Providencia*, were more frequently isolated from in caesarean section-delivered than vaginally-delivered infants up to 12 months of age.

Staphylococcus aureus

Colonization by *S. aureus* was not affected by delivery mode or feeding mode. Colonization by *S. aureus* was much less common in the Italian children than in the British and Swedish children. In the Italian cohort, infants with older siblings were more often colonized by *S. aureus* than children without siblings, but this was not seen in the other groups.

Coagulase-negative staphylococci

Coagulase-negative staphylococci colonized virtually all infants from very early in life. There was a progressive drop in colonization by coagulase-negative staphylococci towards the end of the first year of life. Children who had child-care outside the family at six months of age were less often colonized by coagulase-negative staphylococci compared with infants who were cared for at home.

Enterococci

Enterococci were slightly more often found in infants delivered by caesarean section than in vaginally-delivered infants between one week and six months of age. The caesarean section-delivered infants who were colonized by enterococci also had higher stool population counts than the vaginally-delivered infants colonized by enterococci. This suggests that this bacterial group can expand in the microflora of the section-delivered infants due to absence of certain competitive bacteria in this group.

Infants born to mothers treated by antibiotics during pregnancy were more often colonized by enterococci and, when colonized, also had higher counts of these bacteria. This is further evidence that this bacterial group expands in the absence of other microbes, which are sensitive to antibiotic treatment.

Bacteroides

Colonization by *Bacteroides* was much more frequent in infants delivered vaginally than in those delivered by caesarean section. The delayed acquisition of *Bacteroides* in the section-delivered infants was evident up to 12 months of age, which shows that this bacterial group is not easily acquired if not transferred from the maternal perineal flora at delivery.

Bacteroides colonization differed widely between centres. Thus, the rate of colonization by *Bacteroides* between 2 weeks and 2 months of age in vaginally-delivered infants was approximately 60% in the British cohort, 30-40% in the Swedish cohort, and only 20% in the Italian cohort.

Bacteroides colonization was slightly delayed in infants whose mothers were given antibiotics during pregnancy, and in children exclusively breast-fed for six months. Children in families with pets were slightly more often colonized by *Bacteroides* than children in families without pets.

Bifidobacteria

Bifidobacteria were more common during the first two months in vaginally-delivered infants. After two months, the section-delivered infants had caught up, which shows that bifidobacteria are not only transferred at delivery, but may also be acquired from other sources. Infants exclusively breast-fed for more than six months were no more often colonized by bifidobacteria than infants given mixed feeding or weaned earlier.

Colonization by bifidobacteria was most common in Göteborg and least common in Rome. For example, in one-week-old vaginally-delivered infants the prevalence of bifidobacterial colonization was 82% in Göteborg, 67% in London and 63% in Rome. At one month of age, the corresponding figures were 92% in Göteborg, 83% in London and 73% in Rome.

Lactobacilli

The *Lactobacillus* colonization rate varied strikingly between centres (Table 12). The highest colonization rate was seen in the Swedish cohort. Italian infants were initially less often colonized by lactobacilli, but reached the same levels as the Swedish infants by six months of age. The British infants were infrequently colonized by lactobacilli.

The *lactobacillus* colonization pattern on the genus, species and strain level in the Swedish cohort comprising 116 infants, and the relation between lifestyle factors and *lactobacillus* colonization pattern has been documented in a manuscript submitted for publication in February 2005 (Ahrné et al.). In the Swedish cohort, colonization by lactobacilli was significantly positively associated with breast-feeding (Ahrné et al., in manuscript). No effect was seen between colonization by lactobacilli and delivery mode, siblings or pets in the household.

The dominating *Lactobacillus* species varied with geographical location (Table 13). *L. gasseri* was approximately equally frequent in all three centres (note that Table 13 gives the proportion of different species among positive lactobacillus cultures, hence, a high proportion of a species in the British cohort transforms into a moderate colonization rate by this species). *L. gasseri* occurs in the vaginal flora and has also been reported to be present in breast milk (Martin et al., 2003).

L. rhamnosus was the dominant species in the Swedish cohort. This species colonizes the gastrointestinal tracts from the buccal cavity to the rectum in adult individuals. In Swedish infants, breast-feeding by six months of age correlated positively with isolation of *L. rhamnosus* at the same time-point, which suggests that this species is favoured by breast-feeding (Ahrné et al., in manuscript).

Table 12. The occurrence of lactobacilli (%) in faeces of infants at different ages from Sweden, Italy and England.

Country	1 week	2 weeks	4 weeks	2 months	6 months	12 months
Sweden (n=116)	20	24	35	35	45	19
Italy (n=97)	9	14	21	28	45	23
England (n=108)	7	8	9	16	21	17

Table 13. Proportion of different *Lactobacillus* species in fecal samples from infants of different ages in Sweden, Italy and England.

Lactobacillus rhamnosus

Country	1 week	2 weeks	4 weeks	2 months	6 months	12 months
Sweden	34	40	42	53	46	13
Italy	11	33	26	30	35	21
England	13	22	20	27	20	26

Lactobacillus gasseri

Country	1 week	2 weeks	4 weeks	2 months	6 months	12 months
Sweden	23	40	32	23	4	0
Italy	22	20	40	46	16	4
England	50	44	40	46	16	4

Lactobacillus fermentum

Country	1 week	2 weeks	4 weeks	2 months	6 months	12 months
Sweden	11	3	8	2	8	17
Italy	22	20	9	7	11	4
England	0	0	0	7	11	14

Lactobacillus paracasei

Country	1 week	2 weeks	4 weeks	2 months	6 months	12 months
Sweden	4	0	8	16	30	49
Italy	22	13	13	13	22	37
England	13	22	0	18	24	26

3.5 Strain turn-over

E. coli

The turn-over of *E. coli* strains was substantially higher in the British, compared to the Swedish and Italian infants (Table 14). This reflects the later acquisition of *E. coli* in the two latter, as compared to the former, cohort.

S. aureus

The *S. aureus* strain turn-over was approximately equal in infants colonized by *S. aureus* in the three different cohorts (Table 15). Approximately two different *S. aureus* strains were found per infant, provided they were colonized by the species. Most *S. aureus* strains were resident in the microflora for weeks and months (data not shown). As individual strains of *S. aureus* can persist for extended periods in the infantile microflora, this species must be regarded as a member of the infantile intestinal microflora.

Lactobacilli

The number of lactobacillus strains found in colonized infants was quite low, less than two strains per infants over the first six months of life and approximately two strains per infant over the first year of life (Table 16).

If all children were included in the strain turn-over analysis, whether they ever had lactobacilli in their stool samples or not, the figures decreased to 1.3 strains per six months in Swedish and Italian infants and 0.6 strains over the first six months in the British infants (Table 16).

Table 14. Strain turnover of *E. coli* in children from Sweden, Italy and England. Average number of strains found per infant over a six- or twelve-month period.

<i>Country</i>	<i>6 months</i>	<i>12 months</i>
Sweden	1.5	2.2
Italy	2.2	2.7
England	3.0	4.2

Table 15. Strain turnover of *S. aureus* in children from Sweden, Italy and England. Average number of strains found per infant over a six- or twelve-month period.

<i>Country</i>	<i>6 months</i>	<i>12 months</i>
Sweden	1.5	1.8
Italy	1.4	1.6
England	1.3	1.6

Table 16. Lactobacillus strain turnover. Number of strains found over different time periods in colonized children from Sweden, Italy and England.

<i>Country</i>	<i>1 month</i>	<i>6 months</i>	<i>12 months</i>
Sweden	1.3	1.7	1.9
Italy	1.5	1.9	2.2
England	1.6	1.7	1.8

Table 17. Strain turnover; number of strains found over different time periods in all children from Sweden, Italy and England.

<i>Country</i>	<i>1 month</i>	<i>6 months</i>	<i>12 months</i>
Sweden	0.6	1.3	1.4
Italy	0.5	1.3	1.6
England	0.2	0.6	0.8

3.6 Development of T-RFLP for genetic assessment of intestinal microflora

A sensitive, reliable and robust method to assess the intestinal microflora colonization pattern without culture was developed based on terminal restriction fragment length polymorphism, T-RFLP. The method was published (Wang et al., 2004) and was also presented as part of a PhD thesis (Wang, 2004, University of Lund, Sweden).

To test the applicability of T-RFLP, we compared results obtained by culture of samples from two infants, with the results obtained using T-RFLP and sequencing on the same samples. Sequential stool samples from two infants followed from one week to two months of age were analysed with both methods.

Tables 18 and 19 show a comparison of the results obtained for the two infants by culture on the one hand, and T-RFLP and sequencing, on the other hand. Bacterial names represented in bold are found using both methods, while bacterial names in plain font are only detected by one of the two methods. Bacterial names written in italics are potentially identical with both methods, but the isolates cultured have not been completely identified, in most cases because they died upon subculture.

A slightly larger variety of bacteria was identified by culture than by T-RFLP and sequencing. Culture yielded on average 9.3 (child A, Table 18) and 9.0 (child B, Table 19) different bacterial species or groups, compared to 7.8 and 7.8, respectively, for T-RFLP (Tables 18 and 19). By use of selective media, subdominant bacterial populations of e.g. *E. coli*, *Klebsiella* spp., *Enterococcus* spp. or coagulase-negative staphylococci could be detected, which were missed with T-RFLP as this method detects dominant bacterial populations only. For example, child A had *E. coli* in all samples in counts ranging from $10^{6.7}$ to $10^{9.9}$ CFU/g faeces. Only in samples in which the population counts exceeded 10^9 CFU/g faeces (at 1, 4 and 8 weeks) was *E. coli* also detected by T-RFLP. Thus, culture was superior to T-RFLP for detection of sub-dominant groups, including most anaerobes such as *E. coli*, *S. aureus*, as well as anaerobes of low population numbers, mainly lactobacilli.

Bacteria that instead were missed by culture were aerobic and anaerobic bacteria for which no selective culture media were used, including *Streptococcus* (3 samples), *Eubacterium* (2 samples), *Veillonella* (6 samples), *Ruminococcus* (2 samples) and *Fusobacterium* (1 sample). In addition, a wider spectrum of *Bacteroides* species were detected with T-RFLP than with culture. Thus, T-RFLP was superior to culture for detection of strictly anaerobic bacteria which are difficult to culture.

To summarize, a unique feature of the ALLERGYFLORA project was the application of highly standardized culture techniques, in parallel with development of robust and reliable genetic techniques for non-culture dependent assessment of infantile intestinal colonization pattern. The methodologies could therefore be directly compared, which, to our knowledge, has not been done before. The two methods complemented one another and the combination of both methods will give us a broader and deeper insight into the infantile colonization pattern than obtained previously.

Table 18. Composition of faecal flora as determined by culture, or by T-RFLP and sequencing. Child A

Culture	log CFU	T-RFLP / cloning	Culture	log CFU	T-RFLP / cloning
1w			8w		
B. thetaiotamicron*	9.7	B. thetaiotmicron	Escherichia coli	9.5	Escherichia coli
Gram-positive, oval	9.4	Megasphaera	<i>Gram-negative rod</i>	9.2	<i>Bacteroides merdae</i>
Escherichia coli	9.2	Escherichia coli	<i>Gram-positive cocci</i>	9.0	<i>Ruminococcus gnavus</i>
Enterobacter cloacae	9.1	Bacteroides vulgatus	Klebsella oxytoca	8.8	Clostridium orbiscindens
Enterococcus faecalis	9.0	Sutterella	B. thetaiotamicron	8.8	B. thetaiotamicron
<i>Gram-negative cocci</i>	8.9	<i>Veillonella</i>	B. uniformis/ovatus**	8.6	Bacteroides uniformis
Clostridium difficile	8.9	Clostridium difficile	Klebsiella pneumoniae	8.2	Clostridium
Gram-negative oval	8.8	Clostridium	Clostridium difficile	6.8	Clostridium difficile
Citrobacter freundii	7.3		Enterococcus faecalis	6.6	
2w			6m		
Citrobacter freundii	10.0	Citrobacter freundii	Bifidobacterium	11.0	Bifidobacterium catenulatum
Bacteroides vulgatus	9.8	Bacteroides vulgatus	B. uniformis/ovatus	9.8	Bacteroides uniformis
B. thetaiotamicron	9.8	B. thetaiotamicron	Bacteroides distasonis	9.5	Bacteroides distasonis
Klebsiella oxytoca	9.5	Citrobacter braakii	Escherichia coli	6.7	Bacteroides putredinis
Klebsiella pneumoniae	9.1	Calymmatobacterium	Fungi	6.6	<i>Clostridium celerecrescent</i>
Escherichia coli	8.6		Klebsiella oxytoca	5.9	<i>Clostridium clostridiforme</i>
Clostridium difficile	6.0	Clostridium difficile	Enterococcus faecalis	5.2	<i>Clostridium indolis</i>
CoNS***	5.5		<i>Clostridium</i> 5 different	4.9	<i>Clostridium saccharolyticum</i>
Enterococcus faecalis	4.5				Eubacterium
Lactobacillus gasseri	4.2				<i>Clostridium inocuum</i>
					<i>Clostridium</i>
4w			12m		
B. thetaiotamicron	10.0	B. thetaiotamicron	<i>Bacteroides uniformis</i>	10.2	<i>B. ovatus</i>
Escherichia coli	9.9	Escherichia coli	<i>Bacteroides fragilis</i>	10.0	<i>Bacteroides</i>
<i>Bacteroides</i>	9.8	<i>Bacteroides vulgatus</i>	Gram-positive rods	9.6	Clostridium symbiosum
Klebsiella pneumoniae	8.9	Clostridium	Bacteroides vulgatus	9.5	Clostridium indolis
P. acnes****	7.6		Bifidobacterium	8.7	Bifidobacterium catenulatum
Bifidobacterium	6.1		Escherichia coli	6.7	Bilophila wadsworthia
Clostridium difficile	5.4		CoNS	4.3	Verrucomicrobium spinosum
Lactobacillus gasseri	4.7				Veillonella
CoNS	4.6				Dialister pneumosintes
S. aureus	3.6				Clostridium difficile

Table 19. Composition of faecal flora as determined by culture, or by T-RFLP and sequencing. Child B

Culture	CFU	T-RFLP / cloning	Culture	CFU	T-RFLP / cloning
1w			8w		
Bifidobacterium	10.2	Bifidobacterium	<i>P. acnes</i>	9.4	<i>Bacteroides distasonis</i>
Escherichia coli	9.7	Escherichia coli	Bifidobacterium	9.4	Bifidobacterium infantis
<i>Enterococcus faecalis</i>	9.3	<i>Enterococcus</i>	Bacteroides fragilis	9.1	Bacteroides fragilis
<i>Bacteroides fragilis</i>	8.6	<i>Bacteroides</i>	Enterococcus faecalis	8.5	Enterococcus faecalis
<i>CoNS</i>	7.9	<i>Staphylococcus</i>	Escherichia coli	8.2	Escherichia coli
<i>Clostridium difficile</i>	7.7	<i>Veillonella</i>	<i>CoNS</i>	5.8	<i>Streptococcus mitis</i>
<i>P. acnes</i>	7.6	<i>Streptococcus</i>	<i>Clostridium difficile</i>	5.3	<i>Bacteroides</i>
<i>S. aureus</i>	5.1		<i>S. aureus</i>	4.6	
			<i>Clostridium tertium</i>	4.3	
2w			6m		
Bifidobacterium	10.6	Bifidobacterium	Bifidobacterium	9.2	Bifidobacterium
Bacteroides fragilis	9.4	Bacteroides fragilis	Escherichia coli	8.8	Escherichia coli
Escherichia coli	9.3	Escherichia coli	<i>Enterococcus faecalis</i>	8.5	<i>Enterococcus</i>
B. thetaiotamicron	9.1	B. thetaiotamicron	Bacteroides fragilis	8.5	Bacteroides fragilis
<i>Enterococcus faecalis</i>	8.4	<i>Enterococcus</i>	<i>S. aureus</i>	6.5	<i>Bacteroides uniformis</i>
CoNS	8.2	S. epidermidis	<i>Clostridium septicum</i>	5.9	<i>Bacteroides</i>
<i>Clostridium difficile</i>	6.0	<i>Veillonella</i>	<i>CoNS</i>	4.8	<i>Veillonella</i>
<i>S. aureus</i>	5.9	<i>Streptococcus</i>	<i>C. perfringens</i>	4.5	
			<i>Clostridium difficile</i>	3.5	
4w			12m		
<i>P. acnes</i>	11.8		<i>Bacteroides fragilis</i>	9.8	<i>Bacteroides distasonis</i>
Bacteroides fragilis	10.6	Bacteroides fragilis	Bacteroides vulgatus	9.5	Bacteroides vulgatus
<i>B. uniformis/ovatus</i>	10.6	<i>Bacteroides</i>	<i>Bifidobacterium</i>	8.7	<i>Bacteroides ovatus</i>
Enterococcus faecalis	9.5	Enterococcus faecalis	<i>L. fermentum</i>	7.5	<i>Bacteroides uniformis</i>
Bifidobacterium	9.4	Bifidobacterium	<i>Escherichia coli</i>	7.1	<i>Eubacterium rectale</i>
Escherichia coli	8.6	Escherichia coli	<i>E. gallinarium</i>	6.9	<i>Ruminococcus gnavus</i>
Streptococci	8.6	Streptococcus	<i>Clostridium</i>	6.5	<i>Clostridium fusiformis</i>
<i>Lactobacillus gasseri</i>	7.0	<i>Veillonella</i>	<i>Klebsiella oxytoca</i>	5.4	<i>Veillonella</i>
<i>Clostridium difficile</i>	6.5	<i>Bacteroides vulgatus</i>	<i>CoNS</i>	5.2	<i>Fusobacterium</i>
<i>CoNS</i>	6.3		<i>Clostridium difficile</i>	5.2	<i>Faecalibacterium</i>

Bacterial names indicated in bold are found by both methods. Bacteria indicated by italics are potentially the same, but were not fully identified.

Bacteroides thetaiotamicron*, ** *Bacteroides uniformis* or *ovatus*, *coagulase-negative staphylococci, *****Propionibacterium acnes*.

3.7 Allergy development in relation to colonization pattern

Few bacterial groups showed even a suggestive association with the development of eczema and/or specific or total IgE by 18 months of age. These groups included *S. aureus*, *Bacteroides*, and lactobacilli.

According to the deliverable schedule, the relation between intestinal colonization pattern and allergy should be reported for 50 children per centre, i.e. a total number of 150. The results presented here are based on clinical examination of 116 infants from Göteborg, 106 in London and 79 in Rome. We have, thus, achieved significantly more than scheduled in the application. However, since the records from the clinical examination at 18 months of age were only available for 79 children in Rome and IgE data from 63 children in Rome, all analyses will be redone when all 100 Italian children have undergone the 18 month examination, including total and specific IgE.

S. aureus

Children who developed eczema were more often colonized by *S. aureus* between 1 week and 12 months of age. This was seen in the British and Italian, but not in the Swedish cohort. However, after adjustment for centre, the association between time-to-colonisation with *S. aureus* and clinical eczema at 18 months was not statistically significant at the 5% level, and there were no corresponding associations with total or specific IgE.

Bacteroides

Infants developing eczema at 18 months of age had more often been colonized by *Bacteroides* between 1 week and 12 months of age. However, this association was not statistically significant at the 5% level and there was only a weak tendency that children with increased total IgE and specific IgE to food antigens by 18 months of age had more often been colonized by *Bacteroides* than other infants.

Lactobacilli

Children with high total IgE levels were less often colonized by lactobacilli over the first two months compared with children who had normal IgE levels. This association of high total IgE with time-to-colonisation with lactobacilli was statistically significant at the 5% level, and in the hypothesised direction, but there was no corresponding association of lactobacilli colonisation with specific IgE or clinical eczema. The analysis will be extended to the *Lactobacillus* species level.

Genetic assessment of intestinal microflora

The genetic technique T-RFLP was developed within the ALLERGYFLORA project to assess the colonization by a non-culture dependent technique. The method development was highly successful and resulted in a publication in which the method was described and applied to consecutive isolates of two children followed from 1 week to 12 months of age within the ALLERGYFLORA project. As the microflora of the same infants was assessed by culture, the results of the two methods could be compared, which was shown in a previous section of this report.

We also made a preliminary assessment of whether the T-RFLP technique could be useful in finding differences in early intestinal microflora colonization pattern between infants in relation to later allergy development. To this end, T-RFLP was applied on samples from twelve infants from the Swedish cohort. Five of these infants developed allergy by 18 months of age, defined as an elevated total IgE in combination with at least one allergic symptom (eczema, allergic rhinitis, asthma or food allergy). Seven of the infants were healthy, i.e. they had low total IgE levels, lacked specific IgE against food or inhalant allergens and had no clinical symptoms suggestive of allergy. The clinical characteristics of the infants is shown in Table 20.

Table 20. Clinical characteristics of twelve Swedish infants assessed for microflora diversity by T-RFLP.

Infant no	Total IgE (IU)	Specific IgE to airborne allergens	Specific IgE to food allergens	Allergic eczema	Antibiotic treatment first 6 months	Delivery mode	Duration of exclusive breast-feeding (months)
Allergic infants:							
23A	105	-	+	+	-	vaginal	4
38A	55	-	-	+	+	vaginal	4.5
40A	260	+	+	+	-	caesarean	4
52A	140	-	-	+	+	vaginal	4
71A	24	-	+	+	-	caesarean	5
Healthy infants							
13	2	-	-	-	-	vaginal	0.5
15	6	-	-	-	+	caesarean	5
16	9	-	-	-	+	vaginal	4
31	10	-	-	-	-	vaginal	5
35	8	-	-	-	+	vaginal	4
37	2	-	-	-	-	vaginal	3
47	2	-	-	-	-	vaginal	5

An example of the T-RFLP patterns of two healthy and two allergic infants is shown in Figure 1. The two healthy infants represented in the upper panels were delivered vaginally, while one of the allergic infants was delivered vaginally (38.1 in the figure), the other by caesarean section.

When the number of peaks obtained by T-RFLP were compared between the allergic and the healthy infants, those who later became allergic had fewer peaks than those who remained healthy both at one week of age ($p=0.003$) and at six months of age ($p=0.03$). These figures refer to the T-RFLP pattern obtained after digestion with the enzyme *AluI*. If the enzyme *MspI* instead, the difference was still significant at one week's age ($p=0.048$), but not at six months of age. However, there was still a marked difference in the number of peaks between allergic and healthy infants at six months of age (4 in allergic, 7 in healthy).

A Shannon and Wiener diversity index was calculated (formula, see Materials and Methods section). The diversity index at one week of age was significantly lower in infants who developed allergy by 18 months of age, compared with those who remained healthy ($p=0.005$ for cleavage with *AluI* and $p=0.03$ for cleavage with *MspI*).

In conclusion, we obtained highly interesting indications that the colonization pattern, as assessed by T-RFLP, differed in early age (especially evident at one month of age) between infants who later developed allergy (evident at the clinical examination at 18 months of age) or remained healthy. Currently, we are intensely pursuing this line of research by sequencing and identifying peaks found

in allergic and non-allergic infants. We will also, if possible, extend the analysis to include infants from the three centres.

4. Discussion

The hygiene hypothesis is currently a popular explanation for epidemiological variations in the prevalence of allergy internationally, and over time because it fits with the empirically observed connections between lifestyle and risk of allergy development. In general, the lifestyles which are thought to be characterized by high exposure to microbes in early life are also associated with low risk of developing allergy.

A troublesome fact is that we still do not know which types of microbes are necessary for development of proper immune regulation and when and where these microbes should be presented to the immune system. Also, prior to ALLERGYFLORA, little was known about the relationship between the patterns of normal intestinal colonisation and the established environmental and lifestyle correlates of allergy.

Despite this, administration of probiotics to newborn and young infants as a means to prevent allergy development has already been widely advocated. It should be pointed out that this line of preventive treatment is not based on any observations considering the role of defined microbial groups, species or strains on tolerance development and protection from allergy. Instead, probiotic bacteria already available on the market that have documented effects on completely different conditions, such as childhood acute diarrhea, have been tested for their ability to prevent allergy development. Naturally, there is no reason *a priori* to believe that there would be any connection between these two conditions. In the worst case, probiotic bacteria given in early infancy might instead increase the risk of developing allergy. A chilling observation is that children who have obtained probiotic *Lactobacillus rhamnosus* in their first six months of life have shown somewhat increased risk of developing respiratory allergy by four years of age, compared to non-treated children, despite the fact that their atopic eczema was ameliorated in infancy and early childhood (Kalliomäki et al., 2003). Clearly, there is an urgent need to stringently define which microbial exposure can prevent allergy development and possibly, to identify microbial exposures which may, instead, aggravate the condition.

The ALLERGYFLORA project is an attempt to investigate, in a multi-centre sample, the role of the intestinal microflora in infancy for allergy development. It is the largest study ever performed regarding the establishment of the intestinal microflora in infancy in highly industrialized countries. A genetic-based method, T-RFLP, was also adapted and its performance tested for assessment of the infantile intestinal flora. This technique gave a complementary picture to that obtained by culture in that a greater variety of anaerobic species were identified than with culture. Conversely, culture was more sensitive in detecting sub-dominant populations of bacteria that may have important functions in the microflora, such as enterobacteria, staphylococci and lactobacilli. The results so far obtained in the study has yielded a wealth of information about the determinants of intestinal colonization in infancy and further analysis of the data will continue to yield even more information.

In order to perform the task of pooling three birth-cohorts from three different European countries into a common birth-cohort, an extensive work on method harmonization and calibration work was necessary. We regard the successful method integration as one of the major achievements of the current project. Thanks to the meticulous method scrutinization, training and check-ups, the performances of the three laboratories became sufficiently equal that the results could be pooled. Our results from the checks of culture results of fresh fecal samples distributed between the

laboratories indicated that the results differed little more between the laboratories than within a laboratory, i.e. upon culture and evaluation of a sample by different technicians in the same laboratory. The successfully achieved method compatibility derived from three sources: identical media, typing kits and chemicals used in the three laboratories, extensive training and constant follow-up of performance. In addition, IgE determinations were made in one laboratory in order to minimize variability and extensive discussions were held between the clinicians in order to adjust the methods for clinical assessment.

With a sample size of over 300 infants, and intensive follow-up through the first year of life, we were able, for the first time, to clearly evaluate the effect of a number of lifestyle factors on intestinal colonization pattern, such as delivery and feeding mode, administration of antibiotics to mother and child, pets, siblings and atopic heredity.

In general terms, colonization of the intestinal tract by bacteria is determined by two factors: which bacteria that the baby is exposed to, and whether the bacteria encountered can settle and proliferate in the bowel. The first factor is determined by the degree of environmental exposure to bacteria. For example, infants not exposed to *E. coli* cannot be colonized by *E. coli*. The greater the exposure to *E. coli* via people, animals, food or other sources, the greater probability that the infant will pick up an *E. coli* strain and become colonized.

The second factor, i.e. the colonizing success of bacteria reaching the gut, depends both on individual host factors and on the degree of competition offered by the existing gut flora. A new strain confronted with the gut microbiota can become established, or can pass out without being able to colonize. The greater the variability of bacteria already present, the lesser the chance for a new bacterial strain to become established in the microflora. This phenomenon is termed colonization resistance (Adlerbert et al., 1999).

E. coli and *Bacteroides* represent bacteria which have difficulties to thrive outside the intestinal tract of man or animals. Both are typical faecal bacteria and the presence of *E. coli* is used to signal faecal contamination in foods, water springs and swimming pools. The fact that both *E. coli* and *Bacteroides* are rare in the environment of the newborn infant is reflected by the pronounced association between colonization by these bacteria and delivery mode. Both *E. coli* and *Bacteroides* were much later acquired in infants delivered by caesarean section as compared to vaginally-delivered infants.

We observed a generally delayed acquisition of both *E. coli* and *Bacteroides* in the children examined here, as compared with earlier studies. This was especially prominent in the caesarean section-delivered infants who were not colonized with *Bacteroides* to a similar extent as the vaginally-delivered infants until one year of age. In vaginally-delivered infants, approximately 40% acquired *E. coli* or *Bacteroides* quite rapidly, indicating that they were transferred from the maternal perineal or faecal flora at delivery. But if this was not the case, further acquisition occurred very slowly. This indicates that the circulation of typical faecal bacteria has become very restricted in today's highly hygienic lifestyle.

Other bacteria colonized infants quite without relation to delivery mode. A typical representative were the enterococci, which readily colonized both *sectio*- and vaginally-delivered infants. Enterococci are found in the faeces of man and animals, but their presence in the environment is quite evident, as infants delivered by caesarean section are as rapidly colonized by enterococci as those delivered vaginally. Enterococci are hardy bacteria that survive conventional hygienic measures, such as washing, drying and common disinfectants.

A third group of bacteria appears to have increased as a result of the highly hygienic lifestyle characterizing the Western families examined here. A representative of this group are staphylococci, including *S. aureus*. Staphylococci are typical skin colonizers. All people have coagulase-negative staphylococci on their skin and *S. aureus* is found in moist skin areas (perineum, anterior nostril) in 20-60% of individuals. *S. aureus* has previously not been regarded as a member of the intestinal microflora. In adults, some 20% of all individuals have *S. aureus* in their stools in quite low numbers – 10^3 - 10^4 /g of faeces (Lindberg et al., 2004). A novel observation made in the ALLERGYFLORA cohort was a prominent colonization of infants during the first year of life by *S. aureus*. In the Swedish cohort, 75% of all infants had *S. aureus* at least once during the first year of life. Two findings signaled that this was not merely due to contamination from the skin flora upon sampling – the fact that single strains persisted for many months in the microflora of individual infants, and the fact that *S. aureus* reached quite high population levels, much higher than would have been achieved if the stool sample was simply contaminated by the skin flora of the anus (Lindberg et al., 2004). These two observations led us to believe that *S. aureus* has, indeed, become a frequent colonizer of the infantile gut. We also observed that almost half of the *S. aureus* strains produced one or more toxins with so called superantigen function, either one of the enterotoxins, or TSST-1. The presence of a superantigen-producing *S. aureus* strain in the gut may have profound effects on the immune system, as the superantigens activate 5-30% of all T cells by an interaction with the v-beta region of the T cell receptor, leading to massive activation, cytokine secretion and, in the worst case, shock. Despite this, we did not notice any severe side-effects in the infants colonized by these toxin-producing bacteria (Lindberg et al., 2000). We will continue to evaluate the effects on the immune system of colonization by toxin-producing *S. aureus* after termination of the ALLERGYFLORA project.

It is our hypothesis that *S. aureus* and also coagulase-negative staphylococci have become so frequent and so numerous in the infantile intestinal microflora today due to reduced competition from other members of the intestinal microflora. Anecdotally, an increase in staphylococcal colonization and a decreased colonization by enterobacteria (*E. coli* and the like) has been noted in France in the last decades (Borderon et al., 1996).

Another bacterial group that expands in the microflora in the absence of competitors are the clostridia. These are spore-formers and the spores, which resist heat, drying and disinfectants, are ubiquitous in the environment. Infants delivered by caesarean section were more often colonized by clostridia than were infants delivered vaginally. Preliminary evidence from analysis of the Swedish cohort indicates that infants with atopic heredity are more often colonized by clostridia at one year of age than infants of non-allergic parents. As clostridia are typical of an immature intestinal flora, this interesting observation may indicate that a familial tendency, based on genetics or lifestyle, to have an immature flora, may be connected to an increased risk of developing allergy.

The aim of the present study was to relate intestinal colonization pattern in infancy with later allergy development. We expected to find colonization patterns which were associated with decreased risk of allergy development. Indeed, we found lactobacilli to be associated with decreased risk of having elevated total IgE levels by 18 months of age.

Our hypothesis is that exposure of either intestinal epithelial cells or macrophages or dendritic cells in the intestinal mucosa to lactobacilli promotes maturation of regulatory processes, perhaps regulatory T cells.

The mechanism by which regulatory processes are induced is unclear. However, processing of fed antigens by the intestinal epithelial cell is a necessary step in induction of oral tolerance. It is thought that a tolerogenic form of the antigen is generated during the passage through the small intestinal epithelial cell, a process termed “tolerogenic processing” (Karlsson et al., 2001). Anaerobic bacteria produce short chain fatty acids as end products of their metabolism and it is possible that these metabolites affect intestinal epithelial cells in a manner favouring tolerogenic processing. For example, the presence of a full gut flora, including obligate anaerobes, strongly influences intestinal epithelial cell physiology, as evidenced by increased expression of heat-shock proteins (Ren et al., 2001). It is known that children who develop allergy have evidence of a reduced complexity of short chain fatty acids in their faeces by one year of age compared with healthy children (Böttcher et al., 2000).

Our preliminary evidence with the non-culture dependent technique T-RFLP indicated that infants who later developed allergy had a less complex colonization pattern by one month of age than infants who remained healthy. The microbes of the intestinal microflora constitute the majority of the exogenous antigens facing the developing immune system. It is plausible that a certain minimal degree of variability in the antigens to which the immune system is exposed is needed for proper maturation of the immune system. It is also possible that T-RFLP, which favours quantitatively dominant bacteria, is better for analyzing obligately anaerobic bacteria, compared with quantitative culture of faecal samples. This would be a further indication that anaerobic bacteria are crucial to maturation of tolerogenic processes and protection from allergy development. This line of research will be continued in future projects.

Future studies

An important limitation of the present study was the short time span of observation which was necessary to fit the time-frame of the EU research project. Within the four-year time span of the EU project, we were able to develop methodology, follow the intestinal colonization pattern over the first 12 months in >300 infants, and assess their allergic manifestation by 18 months of age. At 18 months of age, eczema is the major clinical manifestation of allergy, while respiratory allergies generally develop later. Although many of the infants with high IgE and eczema will continue the atopic march and develop other types of allergy, some may instead develop tolerance. Clearly, the children should be observed at later time-points in order to better be able to discriminate between those who will develop persistent allergy and those who will not.

Another limitation of the study was that all three cohorts represented infants born in highly industrialized, privileged countries. It would be immensely useful to study birth-cohorts born in less privileged and developed geographical regions using the same microbiological and clinical methodology. For example, two groups of special interest are infants born in eastern Europe and northern Africa, as well as children raised on small family farms, who are known to have very low allergy prevalence. We are confident that the methodological effort put into the ALLERGYFLORA study will pay back in that the carefully developed methodology can now be applied in new cohorts.

A final goal of the project was to develop reliable methodology for non-culture dependent assessment of the intestinal microflora. After initial attempts to use TTGE, a highly useful strategy was developed in combining T-RFLP with cloning and sequencing. The method was quite demanding, but proved very reliable and exact. Two infants were followed over the first year of life with T-RFLP and cloning analysis of the intestinal microflora. The findings obtained with this method correlated quite well with those obtained with conventional culture but with interesting differences found between the two methods: culture was superior in detecting subdominant bacterial populations, such as enterobacteria, staphylococci and lactobacilli, while T-RFLP was superior in detecting quantitatively dominant obligate

anaerobes. The very promising potential of T-RFLP is that it will allow faecal samples to be collected from cohorts of interest, stored frozen and analyzed at a later time point. In this way, infantile populations e.g. in developing countries can be accurately analyzed with respect to intestinal colonization pattern, even if facilities for culture are not available.

5. Conclusions

The ALLERGYFLORA study is the hitherto largest and most meticulous analysis of the infantile intestinal microflora. By employing highly standardized methodology, we were able to obtain a very high degree of validity and low variability. This was a prerequisite to be able to pool the three birth-cohorts into a common cohort of over 300 European infants. Interesting and novel determinants of neonatal intestinal colonization pattern were observed. However, few bacterial groups associated with risk of allergy development were identified. This might partly depend on the time frame of the project – 18 months is too early to detect many allergic manifestations. We will attempt to follow up the cohort at later time points, provided that financing can be realized.

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