Old Herborn University Seminar Monograph

23. INTESTINAL MICROBIOMICS: NOVEL INDICATORS OF HEALTH AND DISEASE

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ARE STUDIES OF THE GUT MICROBIOME CLINICALLY RELEVANT?

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THE PURPOSE BEHIND THE LECTURE

I was asked by the organizers of this Old Herborn University Seminar to cast a critical eye on the field of research around which this seminar has been organized, namely the study of the intestinal microbiome. My task was to explore the "hard data" upon which assumptions are being made that extend to human health, both with respect to our understanding of the aetiology of certain diseases, their treatment, and the long term maintenance of human health. My motivation in undertaking this task was to explore for myself why so little of the information that has been gathered surrounding the human intestinal microbiome has, as yet, influenced the clinical practice of most allopathic physicians. Rather, information of this type generally is implemented by health care providers who practice "alternative" or "complementary" medicine, and is heavily promoted by companies that sell "health-benefiting" foods such as "yoghurt with active cultures."

SOME EXAMPLES WHERE COMMENSAL MICROBES PROVIDE A "BENEFIT" TO THE HOST

Few of us now doubt that, whether we like it or not, our bodies are the residence of great numbers of microbes, staggering in both numbers and species diversity. What I find remarkable is that our bodies have evolved with the expectation that certain microorganisms will naturally in-inhabit particular niches, whether it is the intestinal tract, the skin surface of our arm, or the corneal epithelium. Without these microbes, certain functions that characterize "health" fail to operate. We call the microbes that normally colonize our body "*commensals*."

Commensal intestinal microbes and epithelial homeostasis

For example, in the mouse large intestine, Gram-negative bacteria are required for the effective repair of the organ following an inflammatory insult (provoked by an oral dose of dextran sulphate) (*Rakoff-Nahoum* et al., 2004). Animals from which Toll-like receptors 2 or 4 have been genetically deleted are more likely than the wild type to experience a destructive DSS induced colitis. A similar phenotype can be demonstrated following DSS administration to animals depleted of colonic bacteria by broad-spectrum antibiotic treatment. The precise mechanism by which luminal bacteria enhance intestinal epithelial repair is not known, although dead bacteria (or LPS) can provide the necessary stimulus (*Rakoff-Nahoum* et al. 2004).

Antimicrobial peptides and harmonious co-existence with intestinal microbes

The mammalian intestine is invested with antimicrobial tools to permit us to live in harmony with our intestinal microflora (*Zasloff*, 2002). In the mouse and in man, antimicrobial peptides produced by the Paneth cells play a major role in protecting the delicate single cell layer that constitutes the intestinal epithelium from lumenal microbes (*Salzman* et al., 2007; *Vaishnava* et al., 2008). Paneth cells normally secrete high concentrations of defensin HD5 and lysozyme into the crypt-well and onto the epithelial surface, generating a protective antimicrobial microfilm. Microbes that access the lumenal surface of the enterocyte are killed rapidly as they attempt to attach to the epithelium. In certain human diseases, like Crohn's disease, this harmony is disturbed, in part as a result of impaired production/secretion of Paneth cell antimicrobial peptides. As a consequence microbes do attach to the epithelium, damage the enterocyte layer, invade into the lamina propria, and provoke a chronic secondary inflammatory response that causes much of the morbidity and pathology associated with Crohn's disease (*Salzman* et al., 2007). Our intestine has been designed to permit us to co-exist with microbes, and these microbes appear to be necessary for normal intestinal epithelial homeostasis.

Systemic bacterial commensals and viral resistance

Certain strains of *Drosophila* were found to be sensitive to infection by RNA viruses, while closely related parental strains were found to be resistant. The resistance trait was passed exclusively by the mother. Review of the origin of the sensitive strains revealed that the sensitive strain had been created from a parental line that had been treated with tetracycline (*Teixeira* et al., 2008). Careful microscopic study of the eggs of a resistant female revealed the presence of numerous cytoplasmic bacteria, not seen in the eggs of females sensitive to viral infection. The microbe was identified as a member of the genus *Wolbachia*. Examination of the tissues of both male and female resistant flies demonstrated the wide spread presence of this "commensal" throughout the animals' body. Sterilization of the viral resistant fly through antibiotic treatment converts it to a sensitive animal. The mechanism by which the presence of systemic *Wolbachia* confers resistance to viral infection is not known, but it does so without apparently diminishing the fertility, lifespan, or "health" of the uninfected fly. This example is presented to illustrate the unequivocal qualitative and quantitative "health benefit" that can be attributed to a "commensal" in certain animals (*Teixeira* et al., 2008).

In addition, I presented examples of bacteria serving a protective function to their host by elaboration of antimicrobial substances that controlled the growth of unwanted fungi. In one example, the embryo of a shrimp (*Palaemon* sp.) has been shown to be covered by a species of *Alteromonas* that secretes an antifungal substance, istatin. Istatin, in turn, prevents the fungus *Lagenidium callinectes* from infecting the embryo, for which it has a particularly great tropism (*Gil-Turnes* et al., 1989). A second example is from the story of the fungus-farming ants. Certain species of "farming ants" inoculate the leaves they harvest with a fungus, which serves as their principal food source. To protect this "farmed" fungus from parasitic microbes that can attack it (especially fungi of the *Escovopis* species), the ants inoculate their crop with a species of bacteria (*Pseudono-*

cardia) which produces a suite of antibiotics that specifically inhibit the growth of the "parasitic" microbial species (*Poulsen* et al., 2007).

GREAT EXPECTATIONS HAVE BEEN SET FOR THE VALUE OF THE KNOWLEDGE OF THE MICROBIOME FOR THE PRACTICE OF MEDICINE

What data exist that would help convince the critical and conservative clinician that human health requires a particular "optimal intestinal microbiome" and that distortion of this optimal collection of microflora can create disease?

In particular, I chose to focus my discussion on the claims made that certain species of intestinal bacteria ("probiotics") are beneficial to health. Highly regarded reviews have suggested that robust data exist to support the claims that:

1. we can effectively manipulate our intestinal flora to increase the proportion of probiotic bacteria, and
2. that such manipulation affords health benefits (*Preidis* and *Versalovic*, 2009).

A widely cited research study is said to have demonstrated that women (with at risk of bearing children with atopic disease) who consumed the probiotic *Lactobacillus rhamnosus* GG during pregnancy had a lower incidence of children with atopic eczema than women who did not consume a probiotic supplement, during the few weeks before delivery and 6 months postnatal (*Kalliomaki* et al., 2003). Careful reading of the original research report, however, would lead one to question the strong conclusion represented in the review, and perhaps, might make one question why the report itself was ever published in the Lancet in the first place. In the study referred to 159 pregnant women were randomly assigned to receive either the probiotic supplement or a placebo beginning 6 weeks before delivery, and continuing postnatal for 6 months. The highlight of the study was that while 24/56 children of mothers on placebo appeared to have atopic eczema at age 4 years, only 15/54 children of mothers receiving probiotics had eczema at that age. On the account of this single finding the paper was titled "Probiotics and the prevention of atopic disease". However, further examination of the data forces one to question the significance of the observation surrounding the apparent reduction in the risk of developing eczema. 10/54 in the treated group developed seasonal rhinitis, compared to 5/56 in the placebo group; skin prick reactivity to the common allergens did not significantly differ between the two groups. From my perspective, these data were unconvincing. Indeed I might worry that I might increase the risk of seasonal rhinitis through probiotic supplementation of mother and infant.

A second widely publicized study examined the impact on upper respiratory infections and antibiotic usage of feeding allergic infants a combination of prebiotics and probiotics (*Kukkonen* et al., 2007). The study is said " to have suggested that effective combinations of probiotics and prebiotics result in sustainable changes in microbial composition and benefits to the human host." (*Preidis* and *Versalovic*, 2009). But a careful review of the original report suggests that the claims exceed the realties reported. In this study, which was randomized and placebo-controlled, pregnant women at high risk for bearing children with allergy were

fed a mixture of 4 probiotics for 1 month prior to delivery, or a placebo. The infants of the treated mothers were then fed the probiotics along with a daily dose of 0.8 g of galacto-oligoscaccaride, a carbohydrate believed to help support the intestinal growth/carriage of the probiotic supplement, while the other cohort received placebo. The infants were followed up for 2 years. The "major" positive effect of treatment was noted between 6 an 24 months. The incidence of respiratory infections was reduced in the probiotic group compared with the control group (93% vs. 97%, p=0.023). The average number of infections was reported to have reduced in the treated vs. control cohorts (3.7 vs. 4.2 infections, p=0.009). And yet, examine the other data presented: similar prevalence of middle ear infections (72% vs. 76%); no effect on incidence of diarrhoeal disease or gastroenteritis; no effect on any infection being followed during the first 6 months while the infants were receiving the supplement; no impact on the incidence of infantile colic. Could one honestly conclude that probiotic therapy affords any benefit to infants at high risk of developing allergic disease?

ALTERING THE MICROBIOME OF A MAMMAL CAN INFLUENCE THE CONCENTRATIONS OF GUT DERIVED METABOLITES FOUND IN BLOOD AND FAECES, BUT IS THIS OF CLINICAL SIGNIFICANCE?

Powerful tools now exist that permit the quantitative and qualitative analysis of organic compounds in the vascular compartment. Several groups have demonstrated unequivocally that many metabolites found in the bloodstream and tissues of mice derive from metabolic conversions carried out by intestinal microbes (*Martin* et al., 2008). If mice are inoculated orally with probiotic bacterial species, distinct differences in metabolites present in blood, liver, and faeces can be correlated with the presence of this probiotic intestinal flora. Does it follow, as these investigators state, that "significant associations between host metabolic phenotypes a nutritionally modified gut microbiota strongly supports the idea that changes across a whole range of metabolic pathways are the product of extended genome perturbations that can be oriented using probiotic supplementation and which play a role in host metabolic health…."

In our studies in the transplanted human small intestine we have discovered that the presence of an ileostomy creates an aerobic environment that favours a microbiome that is enriched in organisms that can tolerate oxygen. In contrast, upon closure of the ostomy, and re-anstomosis of the ileum with the colon, a microbiome that consists of predominantly anaerobic species comes to populate the bowel (*Hartman* et al., 2009). In both settings, the bowel functions normally despite the different population of organism inhabiting the organ. We conclude that the bowel can accommodate different "alternative microbiomic states". Is one state more beneficial than another?

In the final segment of my presentation I reviewed the clinical data gathered to date by Danone, who produce the highly successful product, Activia. Activia is a probiotic-enriched yoghurt to which numerous health benefits have been ascribed. The promotional material used to market this product claims that benefits have been supported by clinical trials. We critically examined one of these published "positive" studies (*Guyonnet* et al., 2007). In a randomized placebo controlled double

blind study, 274 adults with constipation type irritable bowel syndrome were fed once daily for 6 weeks either the Danone product (which contains live organisms of a strain of *Bifidobacterium animalis*) or a heat inactivated control product. The subjects were assessed at week 3 and at week 6, as well as at the start. No clinically significant difference in any parameter measured was presented in the report. Of particular interest was a graph that presented the number of bowel movement/week, measured weekly throughout the course of the study. The graphs of the treated and control groups are indistinguishable. The data suggest that probiotic supplementation conducted in a well-controlled trial provide no significant clinical benefit to those suffering with IBS.

I ended my presentation by asking the participants to consider the following questions as the Seminar exploring the intestinal microbiome of man unfolded:

- With respect to humans: Are there good and bad microbiomes? (Exclude drug-bug interactions).
- If so, how are these two states manifest?
- Should stable differences in a microbiome be considered "Alternative" rather than Good or Bad?
- Prove that by changing an individual's gut microbiome we can impact the health of that individual.

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EXPLORING MICROBIAL COMMUNITIES USING NEXT-GENERATION DNA SEQUENCING PLATFORMS

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SUMMARY

The sequencing of the human genome constituted a starting point in the understanding of human biology at a global scale, yet today there is a growing agreement that human health and disease cannot be understood without considering the microbial communities (microbiome). Now, as the human microbiome project has been launched, a number of international consortiums are starting up with efforts to explore the role of the human microbiota in health and disease. During the last two years molecular microbiology has revolutionized the landscape of microbiology and will continue to do so by providing new solutions for microbe identification and characterization. Next-generation sequencing will open up new areas of research for people in the field of intestinal microbiomics. The high-throughput sequencing platforms we now have access to will hopefully also help to increase our understanding of host-bacteria interactions, immune maturation and mechanisms behind chronic disease development in the gut. The analysis of data resulting from a large-scale sequencing project requires the use of bioinformatics including standard blast analysis, annotation or clustering and assembly competence. In addition, an interdisciplinary approach must be taken on comprising medical, computational, and biotechnology expertise focusing on understanding the human microbial communities and their effect on human health. Application of different "omics"-methods and computational systems biology methods to unique biobanks will be required in order to map out the human microbiome as well as the human cellular machinery interacting it.

TECHNOLOGY PLATFORMS

A human body contains more bacterial than human cells and harbours several microbial ecosystems. The microbes living there and their interactions with the human cells are key components to our health. Malfunctions in these ecosystems clearly lead to everyday health problems currently generating high costs for society and are implicated in health threats such as cancer, type II diabetes, inflammatory bowel disorder, allergy, and obesity. The human microbiome is a complex system with perhaps 1000 microbial species in-

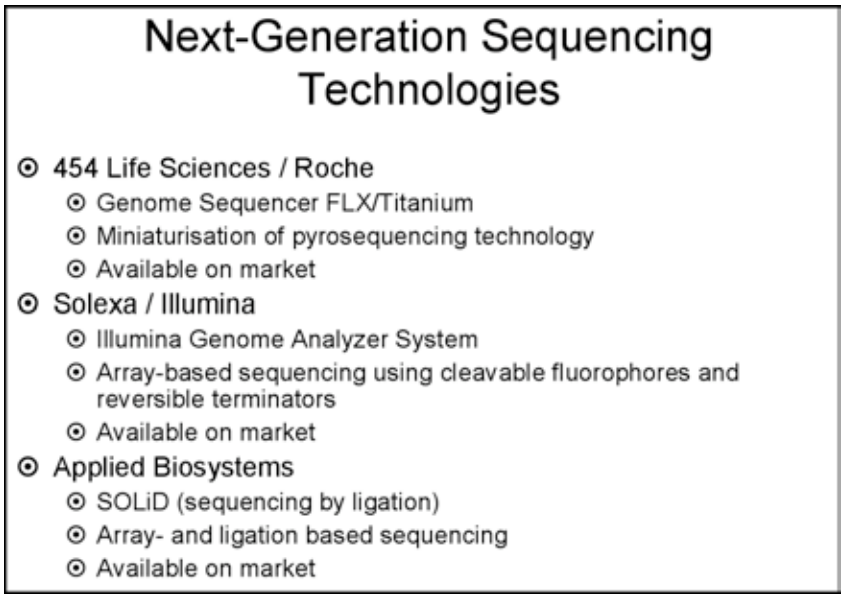

Figure 1: Brief summary of high-troughput technologies available today.

volved, each having around 1000 genes. On top of that, the number of individuals of a species in the microbiome, as well as the pan-genome of a species, is in flux and affected by antibiotic treatment, diet, *et cetera*.

Growing demand in this area of research has fuelled the development of more efficient genomic sequencing methods (*Mardis*, 2008; *Shendure* and *Ji*, 2008). Such methods are already several orders of magnitude more efficient than the Sanger capillary-array electrophoresis machines (for example Applied Biosystems 3730xl) that were used in the human genome project (Figures 2 and 3). Massively parallel DNA sequencing platforms have not only reduced the cost of DNA sequencing but also moved the technology from major genome centres to individual investigators. The new platforms will dramatically accelerate biological and biomedical research, by enabling the comprehensive analysis of genomes to become inexpensive, routine and widespread. Three commercial systems are briefly described in Figure 1.

Roche/454 FLX pyrosequencer

Multiple whole prokaryote genomes can easily be sequenced with the 454 FLX and Titanium systems (*Margulies* et al., 2005; *Ronaghi* et al., 1996). This high-throughput sequencing in real time technology provides long reads (up to 400 base pairs) which facilitates the completion of near-finished draft sequences on a single instrument run. Large-size genomic DNA samples are randomly fragmented into small 300- to 800-base-pair fragments for shotgun sequencing. Addition of adapters to the fragments creates a library of DNA fragments, which is immobilized on DNA capture beads whereafter a PCR amplification takes place in water-in-oil microreactors, resulting in millions of copies of the template. Finally the microreactor is broken and beads carrying single-stranded DNA templates

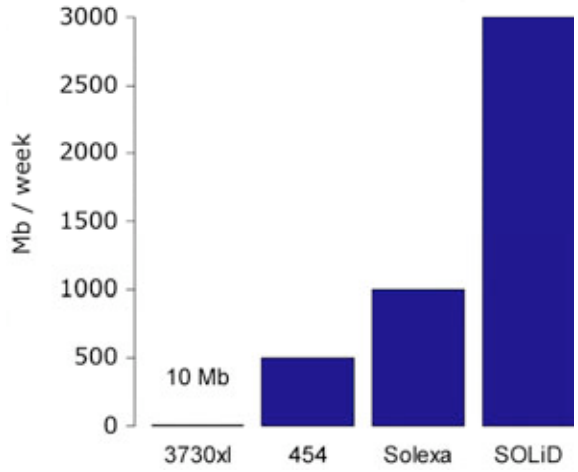

Figure 2: Comparison of yesterdays and todays technologies (Mb output per week)

are individually sequenced on a pico titre plate device. The generated sequences are then assembled into a number of unordered contigs using specific assembler software. Finally a consensus sequence is generated.

The sequencing depth achieved with 454 FLX Titanium sequencing systems ensures accurate characterization of microbial or bacterial diversity, sensitive detection of even rare mutations, and rapid discovery of disease causing agents (*Rothberg* and *Leamon*, 2008). Furthermore, this system for ultra-high-throughput DNA sequencing is used for de novo sequencing and resequencing of genomes, metagenomics and targeted sequencing of DNA regions of interest. The newest version generates up to 500 million bases per 10-hour instrument run. The key-advantage of this technology is read-length (up to 400 base pairs which is necessary in *de novo* assembly and metagenomics). However, a major limitation is that no prevention of multiple

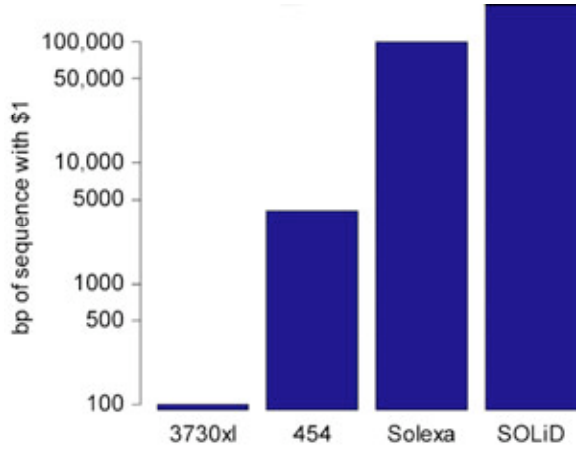

Figure 3: Comparison of different technologies and cost (number of base pairs per US $).

incorporations at a given cycle is provided which leads to homopolymer errors.

The technology has enabled a number of peer-reviewed studies in diverse research fields such as cancer and infectious disease research, drug discovery, marine biology, anthropology, palaeontology and many more. The value of the 454 system for bacterial sequencing applications is underscored by a number of important studies including a *M. tuberculosis* study that resulted in the identification of the first tuberculosis-specific drug candidate in 40 years (*Andries* et al., 2005). The 454 pyrosequencing has so far been the method of choice for exploring the human microbiota (*Andersson* et al., 2008).

Massive parallel 454-tagsequencing targeting the 16S rRNA gene

The most commonly utilized target for microbiome analysis is the ubiquitous gene coding for small sub-unit ribosomal RNA. The 16S rRNA gene (16S rDNA) has historically proved to be the most accurate gene for studies of bacterial diversity, evolution, as well as phylogenetic analysis. The 16S rDNA consists of consensus sequences, universal for all procaryotes, and variable sequences that are specific for particular groups or species of bacteria. Hypervariable sequences, that may be unique for certain strains within a species, are contained within the variable areas. We have developed a novel approach to study the human microbiota using the 454-pyrosequencing platform targeting 16S rDNA (*Andersson* et al., 2008 and Figure 4).

Illumina/Solexa Genome Analyzer

Illumina sequencing technology, or the Solexa platform, allows for selection of any SNP or probe, enabling dense, uniform coverage across the genome and the ability to target any genomic region (*Turcatti* et al., 2008; *Adessi* et al., 2000). This platform is based on massively parallel sequencing of millions of fragments using a reversible terminator-based sequencing chemistry. The technology, together with a software application, allows a scalable system that many consider cost-effective and accurate. It relies on the attachment of randomly fragmented genomic DNA to an optically transparent surface. Attached DNA fragments are extended and bridge amplified to create an ultra-high density sequencing flow cell with ≥50 million clusters, each containing ~1,000 copies of the same template. These templates are sequenced using a four-color DNA sequencing-by-synthesis technology that employs reversible terminators with removable fluorescent dyes. This approach ensures high accuracy and true base-by-base sequencing, eliminating sequence-context specific errors and enabling sequencing through repetitive sequences. After completion of the first read, the templates can be regenerated *in situ* to enable a second >36 bp read from the opposite end of the fragments. A paired-end module directs the regeneration and amplification operations to prepare the templates for the second round of sequencing. Once the original templates are cleaved and removed, the reverse strands undergo sequencing-by-synthesis. The second round of sequencing occurs at the opposite end of the templates, generating >36 bp reads for a total of >3 Gb of data which is an obvious advantage when sequencing large genomes. The short read-length (35 bp) is a limitation and will not provide enough information for 16S identification, but compared to 454 pyrosequencing, homopolymer errors are less of an issue with this technology.

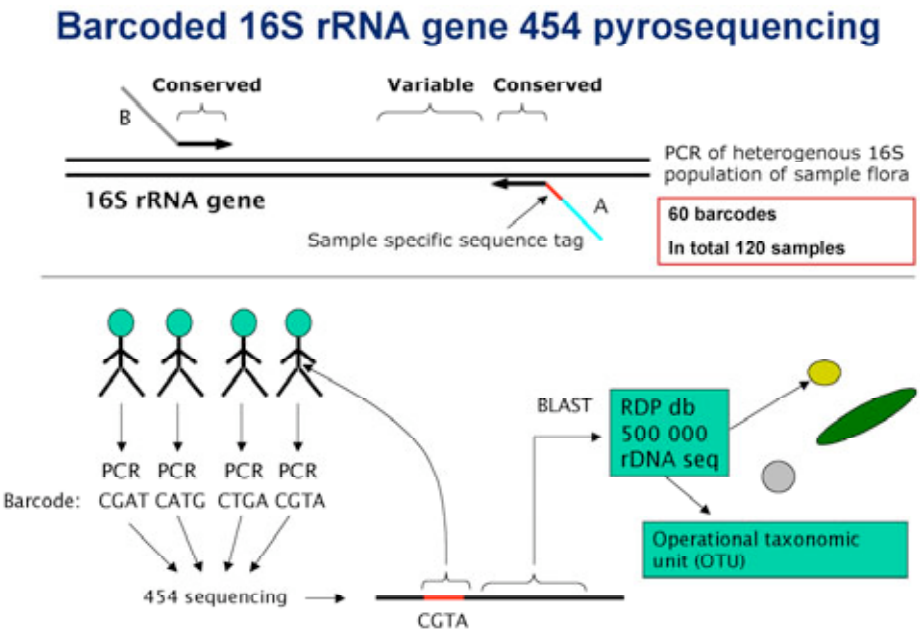

Figure 4: DNA is extracted from each sample and the 16 S rRNA gene contents are amplified by PCR using conserved 16S primers. By using short sample-specific sequence tags, incorporated during the initial 16S PCR reactions, each sequence obtained on the pico titer plate is traced back to its original sample. Thousands of PCR products from each sample are sequenced providing a representative picture of the composition of the microbiota in each individual. The Ribosomal Database Project including more than 500 000 rDNA sequences provides information for species identification (OTU). The capacity of the method is today 120 samples per run at an approximate cost of 80 USD per sample. The 454 pyrosequencing platform allowed us to explore the gut microbiota with a sequencing depth that ensures an accurate characterization. Similar approaches for analyses of the gut microbiota have been reported over the past 6 months (*Huse* et al., 2008; *Dethlefsen* et al., 2008; *Keijser* et al., 2008).

Applied Biosystems SOLiD™ System

Sequencing by ligation generates DNA by measuring the serial ligation of an oligonucleotide. This technology is used in the SOLiD system (*Dressman* et al., 2003; *Shendure* et al., 2005). All fluorescently labelled oligonucleotide probes are present simultaneously and compete for incorporation. After each ligation, the fluorescence signal is measured and then cleaved before another round of ligation takes place. The SOLiD system is a massively parallel genomic analysis platform that supports a wide range of applications. The flexibility of two independent flow cells allows multiple experiments in a single run. The SOLiD system can cost effectively complete large-scale sequencing and with a reference sequence for a microorganism, it is possible to perform comparative sequencing or re-sequencing to characterize the genetic diversity within the organism's species or between closely related species. The throughput is greater than 30 Gb per run but the read-length is limited to 35 bp and consequently not useful for 16S identification.

COMPARISON OF NEXT-GENERATION SEQUENCING TECHNOLOGIES

	454	Solexa	SOLiD
Read length	250-400 bp	25-35 bp	25-35 bp
Reads	1 M	30 M	90 M
Data	500 Mb	3 Gb	30 Gb
Scale-up of # reads	+	+++	+++
Future increase of read length	++	+	+
Access to instruments	+++	+++	+++
Drawbacks	High error rate for homopolymers	Short read length	Short read length
Advantages	Long read length	Easy to scale up	Easy to scale up

CONSIDERATIONS AND CHALLENGES

The cost of sequencing is steadily decreasing, facilitating sequencing of increasing number of microbes and even metagenomics. Many envision that the near future improvements of cost efficiency for DNA sequencing will effectively eliminate sequencing as a bottleneck in biomedical research. The next-generation DNA sequencing platforms will without doubts be applied for a variety of goals within the human microbiome research field. A list of applications that will raise new challenges for experimental design and interpretation of the results have recently been described (*Shendure* and *Ji*, 2008; *Mardis*, 2008; *Rothberg* and *Leamon*, 2008). However, the large amount of data achieved by these instruments must lead to biologically meaningful insights and hopefully also to clinical strategies in the future.

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THE HUMAN INTESTINAL MICROBIOTA; FROM PHYLOGENETICS TO FUNCTIONAL METAGENOMICS

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SUMMARY

The human intestinal microbiota constitutes a complex ecosystem now well recognized for its impact on human health. It does contribute to prevention of colonization by pathogens and maturation of the immune system. Its possible implication in diseases of modern societies, currently increasing in prevalence, has been reported. These include allergies, inflammatory bowel diseases and possible obesity and cancer.

The analysis of the molecular composition of the human intestinal microbiota indicates marked inter-individual variations, which may seem paradoxical considering the high degree of conservation of major functions of the intestinal microbiota such as anaerobic digestion of alimentary fibres. We have characterized a phylogenetic core within the human intestinal microbiota, in terms of composition; i.e. a set of conserved species that could be responsible for major conserved functionalities. Based on culture independent molecular assessments, the current knowledge allows to define criteria qualifying the normal state of the human intestinal microbiota that we call "eubiosis". This further allows to identify specific distortion from eubiosis, i.e. dysbiosis in immune, metabolic or degenerative diseases. Noticeably, Crohn's disease, an inflammatory bowel disease of yet unknown aetiology, is associated with an intestinal dysbiosis with a lower representation of the *Clostridium leptum* group among the Firmicutes phylum. We further showed that the bacterial species *Faecalibacterium prausnitzii* is exerting anti-inflammatory properties *in vitro* and in animal models that could explain its ability, *in vivo* in patients, when it is detectable in the mucosa associated microbiota, to protect patients from post-operative recurrence of endoscopic signs of inflammation 6 months after surgical resection of the ileo-caecal region of the gut.

In confirming the major role of the microbiota in bowel related disorders, especially associated with a disruption of homeostasis, we are currently applying high throughput functional metagenomic screens in order to identify signal molecules and mechanisms of bacteria-host crosstalk. Together with the high resolution description of the human intestinal metagenome as well as explorations at the level of metaproteome and metabolome, these observations will further our understanding of the functional roles bacteria play in maintenance of health and well being in humans. It will open new perspectives for the monitoring and the design of strategies to modulate the microbiota for health.

INTRODUCTION

Microorganisms that colonize man from the very moment of birth on and establish with their host a mutualistic relationship that lasts for the whole life do play major roles that are well recognized today. Yet microorganisms that surround us are still most often perceived as dangerous potential invaders against which we should be ready to fight. This oddly negative perception contrasts with their acknowledged utility for environment, agriculture, industry and mankind.

The human organisms and before man, that of animals have long coevolved over millions of years with microorganisms, leading to the establishment of a mutually beneficial adaptation. Although fine mechanisms or temporal dynamics are not yet known, the host develops a tolerance towards non-pathogenic bacteria that colonize mucosal surfaces and skin and in return, the latter control the development and maintenance of numerous essential functions of their host. The intestinal microbiota contributes to bioconversion of food-born compounds unabsorbed in the upper parts of the digestive tract. Colonization by commensal microorganisms is key to immune development (*Duarte* et al., 2004; *Neumann* et al., 1998; *Souza* et al., 2004; *Oliveira* et al., 2005). The microbiota exerts in addition a direct role of colonization prevention keeping pathogens at levels of population preventing them from expressing virulence (*Freter*, 1983; *Wells* et al., 1988; *van der Waaij* et al., 1971). More recently, a potential role of the intestinal microbiota in regulation of fat storage has been described in mice (*Backhed* et al., 2005) and suggested to operate in man (*Ley* et al., 2006; *Duncan* et al., 2007).

Our vision of the human intestinal microbiota has recently been thoroughly revisited using molecular tools with a marker gene approach based on the use of ribosomal RNA as phylogenetic marker. For an overview of the main stages of the development of the complex microbiota associated to man the reader is invited to consult the appropriate chapter in this monograph. We herein will give an updated view of the intestinal microbiota composition and stability. We will thereafter outline its beneficial roles, and illustrate the association of microbiota dysbiosis and disease. We will finally give perspectives of application of metagenomic exploration of the functionalities of the dominant human intestinal microbiota.

COMPOSITION OF THE HUMAN INTESTINAL MICROBIOTA

Microbial population densities in the human digestive tract reach their maximum values in the colon with 10^{11} bacteria per gram content. It is accepted today that this complex bacterial consortium known as the microbiota is made of hundreds of species in each individual. Yet a thorough description of all intestinal bacteria does not exist for two main reasons:

1. Traditional culture-based characterization (*Finegold* et al., 1974; *Holdeman* et al., 1976; *Moore* and *Holdeman*, 1974) does not allow to take into account more that 30% or so of the microorganisms that can be seen and enumerated by microscopic observation.
2. Species diversity of commensal intestinal bacteria at the level of the planet would be immense. In that respect, the use of molecular tools

indicated that the major part of dominant bacterial species observed in the faecal microbiota of an individual (approximately 80%) are specific of this individual (*Suau* et al., 1999; *Mangin* et al., 2004; *Eckburg* et al., 2005).

If species diversity of the dominant intestinal microbiota gives a faecal print essentially specific of the individual, composition at the level of taxa (genera and/or large phylogenetic groups) highlights consistent components, found in all individuals. Some of these taxa have been known for long and are represented by numerous collection strains; others have been evidenced only recently and via molecular approaches and still have no cultured representative. Culturable genera of the dominant faecal microbiota of adults are *Bacteroides*, *Eubacterium*, *Ruminococcus*, *Clostridium* and *Bifidobacterium* (*Moore* et al., 1974; *Finegold* et al., 1983). Accounting for non-culturable microorganisms allowed to refine this vision and to put it in a phylogenetic framework. The phylum Firmicutes is always highly represented. It comprises the *Eubacterium rectale – Clostridium coccoides*, often the most represented (14 to 31% of total bacteria depending on the studies) (*Franks* et al., 1998; *Jansen* et al., 1999; *Sghir* et al., 2000; *Rigottier-Gois* et al., 2003; *Seksik* et al., 2003). It is composed of species belonging to the genera *Eubacterium*, *Clostridium*, *Ruminococcus*, and *Butyrivibrio*. The phylum Firmicutes also comprises the *Clostridium leptum* group with the species *Faecalibacterium prausnitzii*, *Ruminococcus albus* and *R. flavefaciens*; a group that is also very often dominant (16 to 22% on average) (*Sghir* et al., 2000; *Lay* et al., 2005). Bacteroidetes are represented by genera related to *Bacteroides*. They are always present and share the dominance with the above groups (9 to 42% of total bacteria on average). The phylum Actinobacteria is less consistently detected in dominance, but represents a few percent of total bacteria. It comprises bifidobacteria (0.7 to 10%) and bacteria of the *Collinsella-Atopobium* group (0.3 to 3.7% on average) (*Harmsen* et al., 2000; *Rigottier-Gois* et al, 2003). Enterobacteria are more seldom observed in the top two logs of population in the faecal microbiota (0.4 to 1%), similarly to lactobacilli and streptococci (2%) (*Lay* et al., 2005). Also occasionally found are species related to *Clostridium ramosum*, *Eubacterium cylindroids*, *Phascolarctobacterium*, *Verrucomicrobium* or *Sporomusa-Selenomonas-Veillonella*.

Highly conserved composition traits at the level of phylogenetic groups and phyla on the one hand together with subject specificity at the level of species on the other hand suggest that there exists, on functional grounds, some degree of redundancy between species and the different levels of resolution bring complementary pieces of information.

Finally, bacterial species observed are strictly associated to the intestinal ecosystem. This derives from a long co-evolution with the host (*Ley* et al., 2006) that recent studies of cross-association of microbiota from/with different hosts do confirm (*Rawls* et al., 2006).

At present, phylogenetic reassessment of the human intestinal microbiota has been essentially restricted to the dominant fraction and our knowledge of subdominant bacteria (i.e. below 10^8 per gram stool) may be incomplete and remains restricted to culturable isolates. The ability to isolate and grow microorganisms *in vitro* remains a key step in knowledge building, especially con-

sidering that phylogeny does not inform on the *in situ* activity of microbes. On a more focussed standpoint, the assessment of the contribution of archaea or phages, that may be highly significant in terms of populations, has remained anecdotal up to now.

HOMEOSTASIS OF THE INTESTINAL MICROBIOTA

By definition, a microorganism that colonizes a given niche will persist and multiply without requiring re-inoculation. Dynamics and homeostasis of the intestinal microbiota may be considered in time (for any given individual) and space (i.e. between individuals or intestinal compartments). The global composition of the dominant intestinal microbial community appears conserved between individuals and with time. The same major phyla are present with proportions that vary between individuals but most likely remaining within the same log-unit equivalent in terms of population. Dominant species diversity appears remarkably stable with time for a given individual from day-to-day and even across years (*Zoetendal* et al., 98; *VanHoutte* et al., 2004; *Seksik* et al., 2003) while a large fraction of the dominant species appear specific of the subject. At the level of strains stability is more or less evident depending on the subject (*McCartney* et al., 1996; *Kimura* et al., 1997). Hence the stability observed at the level of groups and species would hide an important rate of renewal at the level of strains. Genomic plasticity may in fact come into play in that respect. It has also been shown that species diversity for subdominant groups (ex. Lactobacilus) is far less stable with time than that of dominant ones (VanHoutte et al., 2004) and that stability of communities is greater in the colon than in the ileum. It must also be considered that lactic acid bacteria brought by the ingestion of fermented food may occasionally have a survival rate during transit that leads to their transient passage in dominance in the small intestine and colon.

For a given individual, modifications of the intestinal microbiota may derive either from colonization by exogenous microorganisms or by modulation of population levels of commensal bacteria. In most cases it will essentially be the consequence of relays in dominance, in response to factors modulating ecological niches. Numerous factors may affect stability of microbial communities - among others transit time, pH, quality and quantity of exogenous substrates and endogenous mucins. Although microbial communities appear ready to deal with changes in ecological settings, it seems difficult to induce durable alterations of established dominant populations, at least in terms of composition. Numerous observations hence illustrate the ability of the dominant intestinal microbiota to resist modification. The administration of an allochtonous strain such as a probiotic or an exogenous non-absorbable substrate such as a prebiotic often lead to transient modifications of microbial equilibrium. Even a major stress such as an antibiotic administration can be followed by a return of the community to its initial dominant species profile within a month or so (*de la Cochetiere* et al., 2005; *Dethlefsen* et al., 2008). This ability to recover its original make-up following a stress, known as resilience, suggests a fine-tuned adaptation of the microbiota to the gut and even to the host that harbours it. This can be linked to the observation that

monozygotic twins have faecal microbial communities that have significantly more closely related patterns than these of unrelated individuals, suggesting that genotype may play a role in the development and structuration of the intestinal bacterial populations.

Analysing the spatial distribution of intestinal microbes as a function of digestive sites is difficult to study; it requires collection of samples within and along the intestine, hence via invasive methods. The preservation of topological relations between bacteria and epithelium is also a challenge. This explains some remaining controversy on this topic.

The luminal microbiota (within the intestinal cavity) has been explored in several ways. The proximal colon luminal microbiota differs from the faecal microbiota of which the composition only represents the distal parts of the colon (*Marteau* et al., 2001). Between the proximal and the distal colon, microbial populations increase overall by a factor of 100 and the increase is essentially due to an increase in strictly anaerobic bacteria. The layer of mucus that covers the intestinal wall constitutes a specific ecological niche. Several studies have shown that the microbial community that colonizes this niche is stable with time and remarkably comparable from the ileum to the rectum for a given individual (*Lepage* et al., 2005; *Wang* et al., 2005). Conversely, species that dominate in the mucus layer differ from dominant luminal species found in faeces (*Eckburg* et al., 2005; *Lepage* et al., 2005).

The ability of commensal gut bacteria to adhere *in situ* to intestinal epithelial cells has so far not been documented in an unequivocal manner. Indirect evidence does exist, derived from the presence of genes encoding adhesions in the genome of strains of *E. coli* able to durably colonize their host. Adhesins could nonetheless contribute to the recognition of mucosal sites and structures or sloughed cells. In ecological terms, a given strain must divide at least as quickly as its offspring's are eliminated in order to maintain itself at a stable level of population in the ecosystem (*Lee* et al., 2004). Hence adhesion to the epithelium does not appear as an absolute necessity, but recognition of sites within the mucus or in the contents would provide a selective advantage for slow growing strains (*Freter* et al., 1983). If adhesion to the epithelium does not seem to be a relevant criterium for commensal bacteria, this property has been associated with intestinal bacteria in patients with inflammatory bowel diseases, and of course many intestinal pathogens.

It remains clear that not all mechanisms involved in maintenance of homeostasis of the human intestinal microbiota are understood to date; noticeably determinants of resistance to change and resilience. It hence is still fully relevant to question the right level of phylogenetic depth and time period for which stability of the ecosystem should be defined. As far as resilience is concerned, it is also reasonable to speculate that a certain level of stress will disturb the equilibrium of the gut ecosystem such that it will be irreversibly perturbed. The threshold above which the human intestinal microbiota loses its ability to return to its original balance is still unknown today.

Finally, parameters of homeostasis will apply to functionalities as much as composition; yet the relevance of these parameters on a functional standpoint is totally unexplored. Functional resistance and resilience of the intestinal microbiota have yet to be determined, as well as the link between phylogeny

and functions. There are only speculations at present on a potential link between "quantity of diversity" and functional resistance and resilience.

EUBIOSIS AND DYSBIOSIS OF THE HUMAN INTESTINAL MICROBIOTA

Based on culture independent molecular assessments, the current knowledge allows to define criteria qualifying the normal state of the human intestinal microbiota that we call "eubiosis". Obviously, emphasis has been put for technical reasons on phylogenetic evaluation of composition, diversity, core species and the dynamics of these over time and space and it is obvious that defining eubiosis will benefit from the addition of functional parameters. This context further allows to identify specific distortions from eubiosis, i.e. dysbiosis, which can be specifically investigated in immune, metabolic or degenerative diseases. We have recently validated the concept in the case of Crohn's disease. Crohn is an inflammatory bowel disease of yet unknown aetiology, that has a prevalence of one per 2000 in European countries. We have demonstrated that Crohn's disease is associated with an intestinal dysbiosis with a lower representation of the *Clostridium leptum* group among the Firmicutes phylum (*Seksik* et al., 2003; *Sokol* et al., 2006, 2008, 2009). We further showed that the bacterial species *Faecalibacterium prausnitzii*, when detectable in the mucosa associated microbiota of the ileum of patients, is protective against post-operative recurrence of endoscopic signs of inflammation 6 months after surgical resection of the ileo-caecal region of the intestine (*Sokol* et al., 2008). We finally demonstrated that *Faecalibacterium prausnitzii* could exert anti-inflammatory properties *in vitro* and in animal models with chemically induced inflammation.

The exploration of dysbiosis may be viewed as a primary step providing key information for the design of strategies aiming at restoring or maintaining homeostasis and eubiosis. Although so far restricted to microbiota composition and/or diversity, dysbiosis has been proposed and in a few cases well documented in irritable bowel syndrome (*Kassinen* et al., 2007), ulcerative colitis (*Sokol* et al., 2008; *Martinez* et al., 2008), obesity (*Ley* et al., 2007; *Kalliomäki* et al., 2008), Type-1 diabetes (*Dessein* et al., 2009; *Wen* et al., 2008), type-2 diabetes (*Cani* and *Delzenne*, 2009), celiac disease (*Nadal* et al., 2007; *Collado* et al. 2009), allergy (*Kirjavainen* et al., 2002; *Björkstén*, 2009), and in cases of infections with *Clostridium difficile* (*Hickson* et al., 2007) or HIV (*Gori* et al., 2008). These observations, just as that of dysbiosis in Crohn's disease above, are not indicative of a causal relationship between microbiota imbalance and onset of the disease. Indeed it is quite reasonable to argue that, once such diseases are declared, owing to the disruption they cause in the immune system and in physico-chemical properties of the intestinal milieu, dysbiosis could in fact be a consequence rather than a cause. In the case of *Faecalibacterium prausnitzii* in Crohn's disease, we nevertheless have a situation in which a deprivation in populations of a normal commensal bacterium, belonging to the most dominant core species of the healthy gut microbiota and potentially anti-inflammatory *in vivo*, will be associated with a reduced ability of the ecosystem to promote a return to immune

homeostasis. It can even be anticipated that a vicious circle is into place combining the detrimental effects of higher bacterial densities close to the mucosa (*Swidsinski* et al., 2005), increased populations of Gram-negative, pro-inflammatory, endotoxin producing bacteria usually subdominant in healthy subjects (*Baumgart* et al., 2007; *Darfeuille-Michaud* et al., 2004), reduced proportions of anti-inflammatory commensals (*Sokol* et al., 2008) and even increased occurrence of protein biomarkers, potentially promoting auto-immune reactivity (*Juste* and *Doré*, personal communication).

The current strengthening of the concept of eubiosis/dysbiosis confirms the major role of the microbiota in bowel related disorders, especially associated with a disruption of homeostasis. It stresses the need to apply the emerging tools of microbiomics to provide diagnostic models and also to identify signal molecules and describe bacteria-host crosstalk mechanisms at play.

HUMAN INTESTINAL MICROBIOMICS

Beyond the phylogenetic level extensively explored so far lie the combined genomes, transcriptomes, proteomes and even metabolomes of the members of the intestinal microbial community. These are known today as the metagenome, metatranscriptome, metaproteome, .. and all-together the human intestinal microbiome. Since the vast majority of dominant intestinal bacteria are not yet cultured to date, the genomic content of this microbial community and its derived components at the various levels of omic integration are essentially unknown. Metagenomics is emerging today as this most powerful approach to characterize the repertoire of genes of any complex microbial setting, independent of the culturability of its components. The development of very high throughput sequencing technologies is further contributing to this development. In practical terms, the microbial community may be extracted from its environment and its DNA purified and/or cloned in order to determine its sequence. The complete gene repertoire of culturable and non-culturable dominant microbes can hence be obtained. This further offers the possibility to design high throughput profiling tools informative at the level of functional potentials of the complete community. In addition, cloning of genome fragments of intestinal bacteria in large insert metagenomic libraries allows to use functional screens in order to seek functions of non-culturable bacteria after heterologous expression in *E. coli*, giving access to yet totally unexplored biological resources. Beyond its innovative character, the potentials of metagenomics have already largely been documented (*Riesenfeld* et al., 2004; The new science of metagenomics: revealing the secrets of our microbial planet, National Research Council of the National Academies, The National Academies Press, Washington DC, USA, 2007).

The first developments of the metagenomics approach applied to the human intestinal microbiota have focussed on diversity of the microbial community (*Manichanh* et al., 2006, 2008) and the gene repertoire for a few subjects (*Gill* et al., 2006; *Kurokawa* et al., 2007). Functional metagenomics applications have remained confined to the soil ecosystem (*Williamson* et al., 2005) and animal guts (*Beloqui* et al.,

2006). We initiated its application to the human intestinal context (*Gloux* et al., 2007). Although still in its infancy, this approach brings major promises for an improved understanding of microbe-food, microbe-host and microbe-microbe interactions.

The microbiomic exploration of the human intestinal microbiota is hence ongoing thanks to several programs such as the European Commission-funded program MetaHIT (http://locus.jouy.inra.fr/metahit/), and the French Agency for Research-funded program GMGE Micro-Obes (http://www.inra.fr/micro_obes), as well as the NIH Roadmap programs (http://nihroadmap.nih.gov/hmp/) and many others worldwide. At the international level these programs are structured within the International Human Microbiome Consortium (IHMC) co-chaired by the NIH and the European Commission. These programs will deliver a huge mass of information that will in turn allow to identify conserved and variable genomic and functional traits of the ecosystem, to describe those specific to the gut environment and bearing the best diagnostic and/or prognostic potential, to reconstruct the metabolic food-chain of the microbial community, and to start describe ecotypes and model their relationships in a systems' ecology endeavour.

CONCLUSION

Application of molecular ecology tools to the intestinal microbiota has allowed very significant improvements in our understanding of this ecosystem in terms of composition and dynamics of species diversity. The single gene approach based on ribosomal RNA as a universal phylogenetic marker had nevertheless left aside the functions microorganisms exert in their environment.

It has become possible to sequence combined genomes of complex microbial communities giving access to their potential activities. The following steps towards environmental transcriptomics, expressed proteins, activities and metabolites are being taken. The global functional exploration of the human intestinal microbiota is hence underway, with perspectives as large and fascinating as these of the former decade.

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METAGENOMIC APPROACHES TO UNRAVEL THE COMPOSITION AND FUNCTION OF THE HUMAN SMALL INTESTINE MICROBIOTA

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SUMMARY

The small intestine microbiota remains largely unexplored, which is a consequence of the poor accessibility of this ecosystem. Nevertheless, this part of the intestine is of great importance for physiological homeostasis of the host. Not only is the small intestine the major site of nutrient absorption, but it also provides the most important mucosal immune organ of our body. The interaction of the small intestine mucosa with the residing luminal and adhered microbial populations is bound to represent an important cross-talk repertoire that is importance for health. The review presented here, provides the insights obtained in small intestinal microbiota composition and function. The model system used for these studies is that of ileostoma-individuals that lack a colon but have a normally functioning small intestine. The studies encompass phylogenetic community composition analyses, as well as metagenome and metatranscriptome analyses. The results presented highlight several aspects of small intestine community structure and function that generate a clear and comprehensive view of this habitat and the selective forces that shape its residing microbial community.

INTRODUCTION

The human gastrointestinal (GI) tract is inhabited by a consortium of microorganisms that is strongly dominated by bacteria and is referred to as gastrointestinal microbiota (*Guarner*, 2006; *Leser* and *Molbak*, 2009). Besides bacteria the presence of Eukarya (*Scanlan* and *Marchesi*, 2008) and Archaea (*Dridi* et al., 2009; *Eckburg* et al., 2005) in the human GI tract has been reported, albeit with relative low abundance and diversity. Moreover, an impressive viral community has been detected in the human intestine using meta-analyses and revealing 1,200 viral genotypes in faeces obtained from adult subjects (*Breitbart* et al., 2003). Based on their dominance in the intestinal ecosystem, most attention has been given to the bacterial communities in this system. Traditional approaches have employed cultivation as the main method to study the microbial community in the human intestine. However, the application of molecular methodologies to unravel bacterial

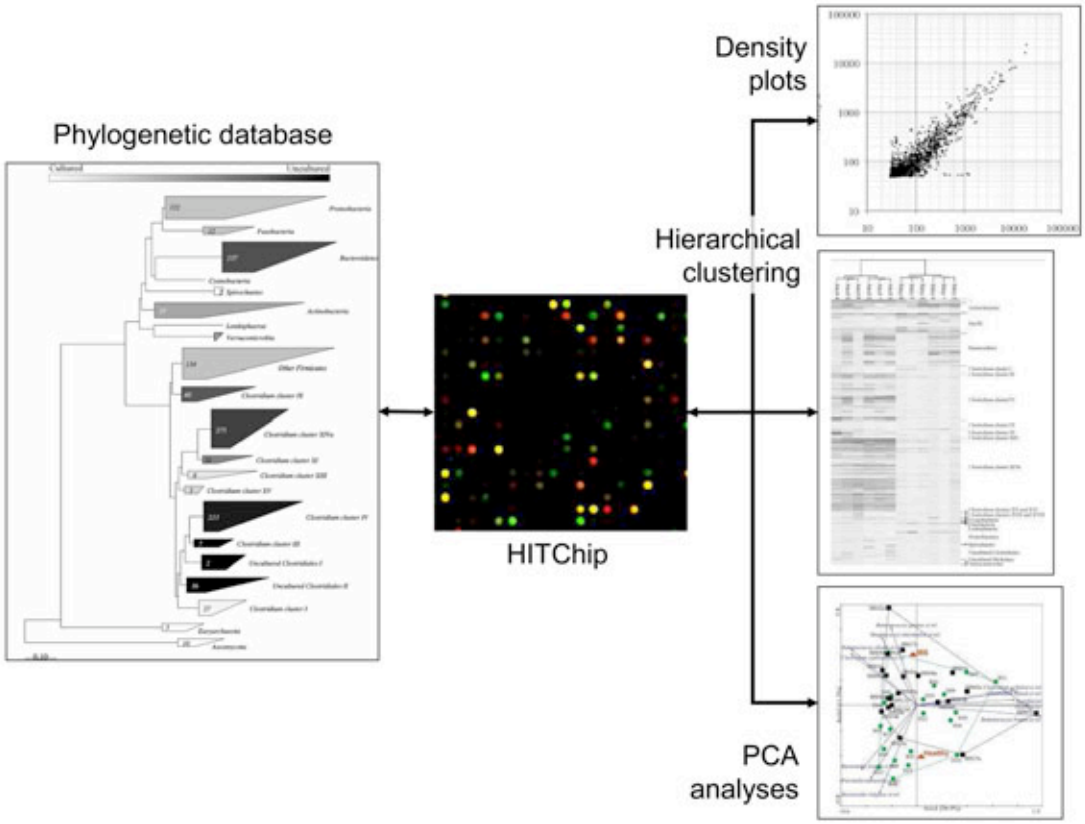

Figure 1: Schematic representation of the application of the Human Intestinal Tract Chip (HITChip) designed at Wageningen University (*Rajilic-Stojanovic* et al., 2009), which is based on the Agilent format slides, containing probes designed for the variable regions 1 and 6 (V1 and V6) of the 16 S rDNA sequences of more than 1200 non-redundant bacterial OTUs. Data acquired by HITChip provide direct phylogenetic connection to the underlying probe-phylogeny assignment database. A series of processing scripts enables effective and (semi-) quantitative interpretation of the array data in terms of microbiota composition at various levels of phylogenetic depth (i.e., from phylum down to species), which can be visualized using various graphical modules, and can be analyzed by a variety of statistical and/or clustering software packages that are linked to the array database.

community structure in the human intestine that has been applied since approximately 15 years revealed that cultivation approaches have severely underestimated the bacterial population diversity and underlined that the majority of the bacterial species residing in this habitat appear to be uncultured to date (*Zoetendal* et al., 2008).

The highly diverse bacterial community residing in the human GI tract (Figure 1) is dominated by phylotypes belonging to the Firmicutes, Bacteroidetes, and Actinobacteria (*Backhed* et al., 2005; *Eckburg* et al., 2005; *Rajilic-Stojanovic* et al., 2007). Although Firmicutes are found in the intestine of all mammals, each mammalian species harbours a distinct microbial composition showing relatively limited variation at intraspecies level (*Ley* et al., 2008a). Intestinal microbiota composi-

tions of different mam-mals can be clustered by dietary habits revealing distinct community structures in carnivores, omnivores and herbivores, which can be roughly characterized by increasing microbiota diversity, respectively. In addition, discriminative clustering could be achieved on basis of host phylogeny and cognate intestinal anatomy. Consequently, it is not surprising that the human intestinal microbiota composition resembles that of omnivorous primates (*Ley* et al., 2008a,b).

Recently the interest in understanding intestinal community structure and function has increased on basis of observations that indicate correlations between the human intestinal microbiota and human health and disease. This field may eventually provide microbiota-based diagnostic markers for health and disease, while such insight may also offer avenues towards diet or microbial intervention strategies to prevent or treat human diseases via modulations of the intestinal microbiota. However, the success rate of these latter possibilities will depend on the causal relations underlying the observed correlations between intestinal microbiota and health and disease.

MOLECULAR METHODS TO UNRAVEL BACTERIAL COMMUNITY STRUCTURE IN THE HUMAN INTESTINE

Culture independent molecular methods to determine bacterial community structure commonly target the universal bacterial phylogenetic marker, 16S ribosomal rRNA (rRNA) or its encoding gene. Frequently applied 16 S rRNA methods to assess (quantitative) composition and diversity of the microbiota on basis of 16 S rRNA include classical cloning and sequencing, DGGE/TGGE, FISH, and qPCR. These methods provide different degrees of sensitivity, selectivity and phylogenetic resolution and have been employed to determine bacterial-community structures, or detect and/or quantify specific bacterial groups within a variety of samples derived from the human intestine. Both these methodologies as well as their overall results have been reviewed recently (*Zoetendal* et al., 2004) and will not be discussed here. Nevertheless, two emerging technologies, i.e. phylogenetic microarray analysis and barcoded pyrosequencing, will be reviewed here since they offer novel, high-throughput in depth composition profiling possibilities.

Phylogenetic microarrays are commonly constructed by printing 16S rRNA-targeting oligonucleotide probes on a carrier surface (in many cases glass slides). This platform enables high-throughput, in depth, semi-quantitative characterization of microbial communities (*DeSantis* et al., 2007; *Paliy* et al., 2009; *Rajilic-Stojanovic* et al., 2009; Figure 1). An obvious constraint of phylogenetic microarrays is their restriction to detect phylogenetic groups that are represented in the array design (*Palmer* et al., 2006), which may provide incomplete composition impressions in specific samples (as an example see below). Therefore, array design should preferably be updated on a regular basis to incorporate newly identified bacterial groups that inhabit the target niche, e.g. the human colon. An alternative methodology that holds great promises for high-throughput analysis of microbial composition in human intestinal samples (or any other environmental sample) is designated barcoded 454 pyrosequencing (*Andersson* et al., 2008). This method is based

on sequencing-based *de novo* community profiling, and consequently is not restricted to 16S rRNA sequences that are known beforehand. This technology is compatible with high-throughput analyses and provides relatively high phylogenetic resolution. Pyrosequencing has been employed to unravel the microbial community structure in various samples obtained from humans, including the intestinal tract (for a review see: *van den Bogert* et al., 2010). Importantly, deep pyrosequencing and phylogenetic microarray analysis of the microbial community of faecal samples generated comparable results (*Claesson* et al., 2009; *van den Bogert* et al., 2010). Pyrosequencing-data interpretation is not trivial due to the huge amounts of sequences generated and requires stringent sequence quality control and effective taxonomic profiling, interpretation and visualization software suites. The level of sensitivity of the pyrosequencing method depends on the amount of the sequences generated for an individual sample, and probably requires a 10- to 100- fold excess of the depth that is targeted for.

Pyrosequencing, like other PCR-based profiling technologies suffers from the potential biases introduced by the amplification-primers used as well as by intrinsic biases of the DNA amplification reaction itself. Due to the continuous expansion of the 16S rRNA database, primers (and probes for application in FISH) tend to become outdated and therefore require constant updating to ensure appropriate coverage of the targeted microbial population (*Baker* et al., 2003). The PCR amplification of highly conserved genes like the 16S rRNA gene intrinsically suffers the risk of chimera formation, which may contaminate the databases with biologically irrelevant sequences that are falsely assigned to specific bacterial groups (*Ashelford* et al., 2005). Recently the field of random shot-gun sequencing of environmental DNA (metagenomics; *Handelsman,* 2004) has expanded drastically as a consequence of the development of extreme-throughput sequencing technologies (454-Titanium; Illumina and Solid), which will enable the development of alternative methods for community composition profiling that are based on function pattern determinations rather than the single marker 16 S rRNA gene. A landmark achievement in this field is the recent release of a human intestine microbiota reference gene-set that contains more than 3 million bacterial genes discovered within the intestinal community. This large amount of genetic information of the human intestine microbiota offers an unprecedented level of resolution for function based profiling for ecosystem communities (*Qin* et al., 2010). For example this database can be employed as a reference template for profiling and pattern recognition using high-throughput short-sequence DNA information as can be obtained from next-generation sequencing technologies like Illumina or Solid.

THE LARGE AND SMALL INTESTINE OF HUMANS

The bacterial community is not evenly distributed over the different regions of the human gut; it increases in density along the longitudinal axes. The bacterial populations of the stomach are relatively small (10^3-10^4 bacteria per gram of contents), which is due to the harsh conditions encountered in this habitat such as very low pH values (approximately 2.5 in humans) and

other antimicrobial factors (*Guarner*, 2006; *Leser* and *Molbak*, 2009). The diversity of stomach microbiota is low, and merely 128 phylotypes (or operational taxonomic unit: OTU) could be recovered from 23 individuals (*Bik* et al., 2006).

Once entering the small intestine, bacterial densities increase to approximately 10^4 to 10^5 bacteria in the jejunum and 10^8 or even more bacteria in the terminal ileum. Climax community densities are reached in the large intestine where more than 10^{11} bacteria per gram contents have been reported (*Guarner*, 2006; *Leser* and *Molbak*, 2009). Based on their accessibility, faecal samples are often used to study the microbiota in the large intestine (*Huys* et al., 2008). However, several studies have shown the marked difference between bacterial community structures in faecal samples as compared to those adhered to the colonic mucosa (*Eckburg* et al., 2005; *Lepage* et al., 2005; *Zoetendal* et al., 2002). The large intestine microbial community is highly complex and has been estimated to encompass a complexity that spans at least 500 phylotypes (*Eckburg* et al., 2005), which predominantly classify within the phyla Firmicutes, Bacteroidetes, and Actinobacteria. The large intestine microbiota composition appears to be very individual-specific (*Zoetendal* et al., 2008) and displays considerable stability over time and resilience following antimicrobial interventions like antibiotic treatments etc. (*Matsuki* et al., 2004; *Rajilic-Stojanovic* et al., 2009; *Zoetendal* et al., 1998). Nevertheless, a recent study identified a potential group of potential core microbial phylotypes that inhabit the majority of humans at high relevant abundance (*Tap* et al., 2009). Analogously, faecal microbiota profiling by phylogenetic microarrays, revealed a set of responding probes that were shared among the individuals (*Rajilic-Stojanovic* et al., 2009). Inversely, the vast majority (~80%) of the detected phylotypes appears to be host specific (*Tap* et al., 2009). Despite the observed composition variation among the intestinal microbiota in human individuals, metagenomic analysis has recently indicated that there appears to be a remarkable functional congruency in these different microbial communities (*Turnbaugh* et al., 2009). Analogously, abundance profiling of the reference gene set of the human microbiome (*Qin* et al., 2010) in individuals reveals that the majority of genetic functions is conserved among the faecal microbiota of individuals, while a portion (~10% of the complete 3.3 million genes identified) of specific genes is actually shared among all individuals and can be regarded as a core metagenome. Moreover, gene frequency analysis comparing human intestine metagenome datasets and whole bacterial genomes or metagenome data sets obtained from other environmental niches led to identification of gene sets that are specifically enriched within the human faecal metagenome (*Qin* et al., 2010).

Recent research has exemplified that a healthy human host and its intestinal microbiota coexist in a homeostatic relationship (*Hooper*, 2009; *Leser* and *Molbak*, 2009; *Macpherson* and *Harris*, 2004). The intestinal microbiota benefits from a stable environment and nutrient supply that are provided in the intestinal tract, while the host gains products from microbial fermentation conversion of host indigestible components into short chain fatty acids (SCFA; acetate, propionate, butyrate; 10% of our energy requirement), vitamin K and B12 production and protection against potential pathogens (*Guarner*, 2006; *Leser* and *Molbak*, 2009; *Macpherson* and *Harris*, 2004;

Neish, 2009). Overall, the intestinal microbiota composition and activity patterns may have a pronounced effect on human health and several studies indicate that certain health disorders are associated with deviations in aberrations in the intestinal microbiota composition and/or function (for a recent review see: *Leser* and *Molbak*, 2009). Extending our knowledge of this microbial ecosystem therefore holds great promise for future interventions that aim to prevent or treat certain diseases or disorders though modulation of the intestinal microbiota community.

MODELS TO STUDY THE SMALL INTESTINE MICROBIOTA

Contrary to the large intestine, our knowledge of the microbiota that inhabits the human small intestine is limited. This is largely due to the sampling difficulties for this region of the GI tract that is notoriously difficult to access. Consequently, the small intestinal microbiota studies to date depended largely on biopsy specimens obtained during (emergency) surgery (*Ahmed* et al., 2007) or samples collected from sudden death victims at autopsy (*Hayashi* et al., 2005). Microbial analysis of biopsies from the jejunum and the distal ileum revealed a relatively low bacterial diversity in the jejunum mucosa with a microbial community dominated by *Streptococcus* sp. while a predominance of *Bacteroidetes* and *Clostridium* clusters IV and XIVa (according to the phylogeny proposed in *Collins* et al., 1994) was identified for the distal ileum (*Wang* et al., 2005). However, as a consequence of the relatively extensive procedures required for obtaining these samples, they may not represent the true small intestinal microbiota of a healthy individual and do not provide insights into population dynamics (*Booijink* et al., 2007).

One alternative to obtain small intestinal samples, which circumvents the sampling difficulties associated with the small intestine, makes use of individuals that underwent surgical removal of the colon due to cancer or inflammatory bowel disease (IBD) and as a result have the terminal ileum connected to a stoma. This ileostoma provides a unique opportunity to non-invasively and repetitively sample the contents of the terminal ileum (*Booijink* et al., 2007, 2010). A recent study indicated that the microbiota in the effluent samples from these ileostomy subjects does not represent that of the terminal ileum in healthy subjects due to the penetration of oxygen (*Hartman* et al., 2009). Although this study seems to contradict with recent findings by Booijink that showed high abundance of strict anaerobes in ileostomy effluents (*Booijink* et al., 2010), preliminary investigations in our laboratory with an orally introduced catheter revealed microbial communities were enriched in *Streptococcus* and *Veillonella* (belonging to *Bacillus* and *Clostridium* cluster IX, respectively) in jejunal and proximal ileal regions of the small intestine, while abundance of *Bacteroidetes* and *Clostridium* cluster XIVa were dominating in the terminal ileum (Figure 2), resembling the microbiota in ileostoma effluent and the colon, respectively. These results suggest that ileostoma effluent is probably not an appropriate reflection of the terminal ileum lumen, but more proximal regions of the small intestine. Since these regions are among the first to interact with dietary components, it seems that the ileostoma model system provides an excellent model to study these early

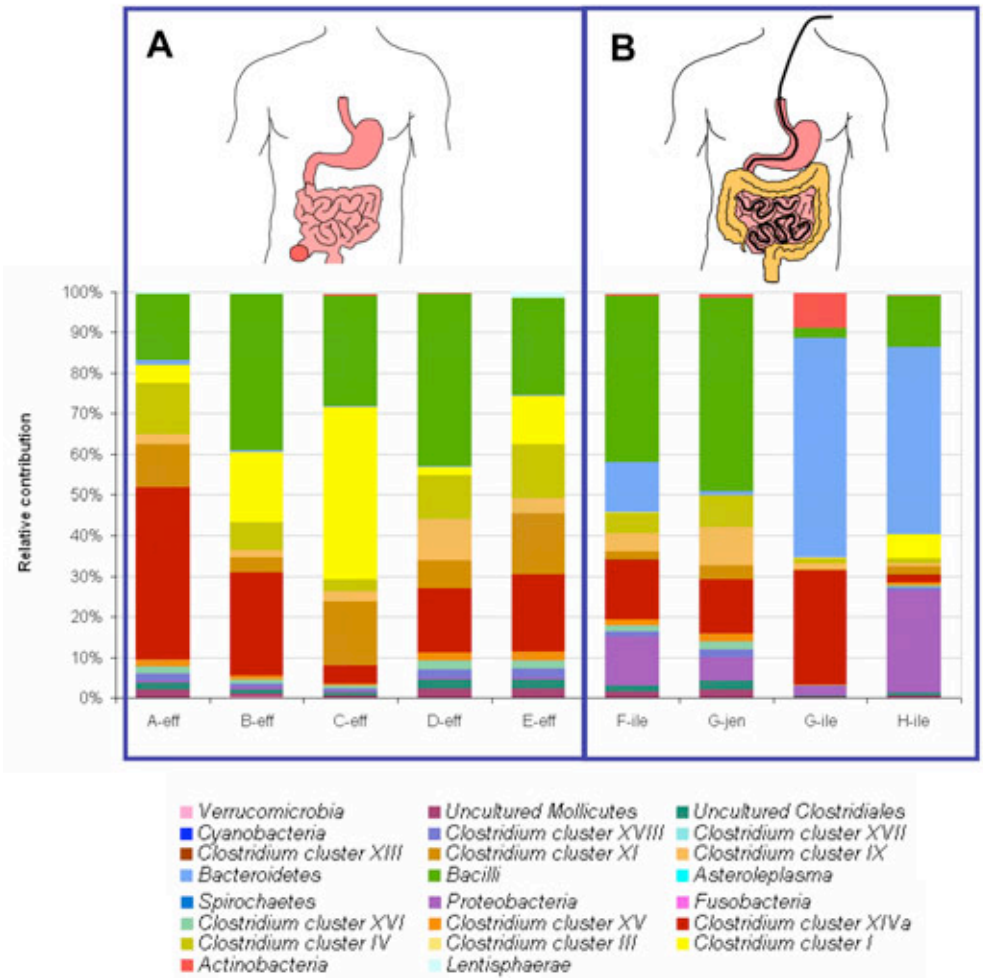

Figure 2: Schematic representation of the two small intestinal models employed, i.e., the ileostoma model (A) and the extended oral catheter sampling (B). The relative contributions of detected phylogenetic groups are shown for ileostoma effluent of 5 ileostoma individuals (A-F), and catheter obtained small intestinal samples of 3 healthy individuals (F, G, and H) from the indicated regions of their intestine (ileum; ile, and jejunum; jen) (adapted from *van den Bogert* et al., 2010).

microbiota-diet-host interactions (Figure 2). The terminal ileum of normal individuals appears to resemble a mixed population of jejunal and proximal-ileal communities and those from the colon, which may be maintained through colonic reflux into the terminal ileum.

SMALL INTESTINAL MICROBIOTA AND ITS INTERACTIONS WITH DIET AND HOST MUCOSA

The small intestinal microbiota is the first microbial community of the intestine that interacts directly with the diet. Consequently, the small intestine microbiota has to compete with the absorptive capacity host mucosa for the

available carbon and nitrogen sources. Moreover, the physico-chemical conditions of the small intestine are harsh from a microbe's point of view, e.g., residence times are short and exposure to bile acids and host-digestive enzymes is constant. Therefore, it can be anticipated that the small intestine microbiota community is strongly influenced by changes in dietary composition that address microbial capacities differentially (*Booijink* et al., 2010). These dietary interactions can be expected to be more direct and more pronounced as compared to the large intestine, where most readily utilizable components of the diet have already been removed by absorption by the host and by the small intestine microbes. As a consequence the large intestinal microbiota focuses on the materials that have escaped the digestive capacities of the host and the small intestinal microbiota, and is commonly regarded as a large fermentative organ.

The small intestine harbours a large proportion of the body's immune cells, and thereby plays a prominent role in development and maintenance of appropriate immune homeostasis in newborn and adult mammals, respectively (*Brandtzaeg*, 1998; *MacPherson* and *Harris*, 2004). The intestine's mucosal tissue provides a highly sophisticated barrier that prevents inflammation of the underlying tissues by a complexly controlled and highly flexible innate and adaptive mucosal immune system. In this system, there is a particularly important role in induction and regulation of mucosal immunity for the immune-dedicated GALT (gut-associated lymphoid tissue) system, including important roles for Peyer's patches and mesenteric lymph nodes. The density of these dedicated immune sensing and modulation systems is much higher in the small intestine as compared to the large intestine, indicating that the small intestinal microbiota can be considered a prominent driver of the host-immune system.

It seems plausible that the development of functional foods that aim to modulate the host's immune system are more likely to act in the small intestine, and may include the interaction with the endogenous microbiota in this region of the intestine. Based on the above, it is of utmost importance to improve our understanding of the small intestine microbiota composition and function in relation to diet, and how diet-associated changes of this community may affect the overall functioning of the host's immune system.

SMALL INTESTINE METAGENOMICS

To obtain insight into the genetic potential within the small intestinal microbiota, in our laboratory we have constructed a large-insert (fosmid cloning vector) metagenomic library from ileostoma effluent obtained from a healthy individual who has had the stoma for more than 20 years and does not need medication for stoma-related problems. Since previous work had established that the ileostoma effluent microbiota fluctuates over time (*Booijink* et al., 2010), the metagenome libraries were constructed from four different samples of this one individual to encompass as much of the overall diversity of its microbial community as possible. The overall fosmid metagenome library constructed encompassed 25,344 clones that on average contain approximately 25-30 kb of insert DNA, indicating that the overall library contains more than 700 Mb of genetic material from the microbes within this

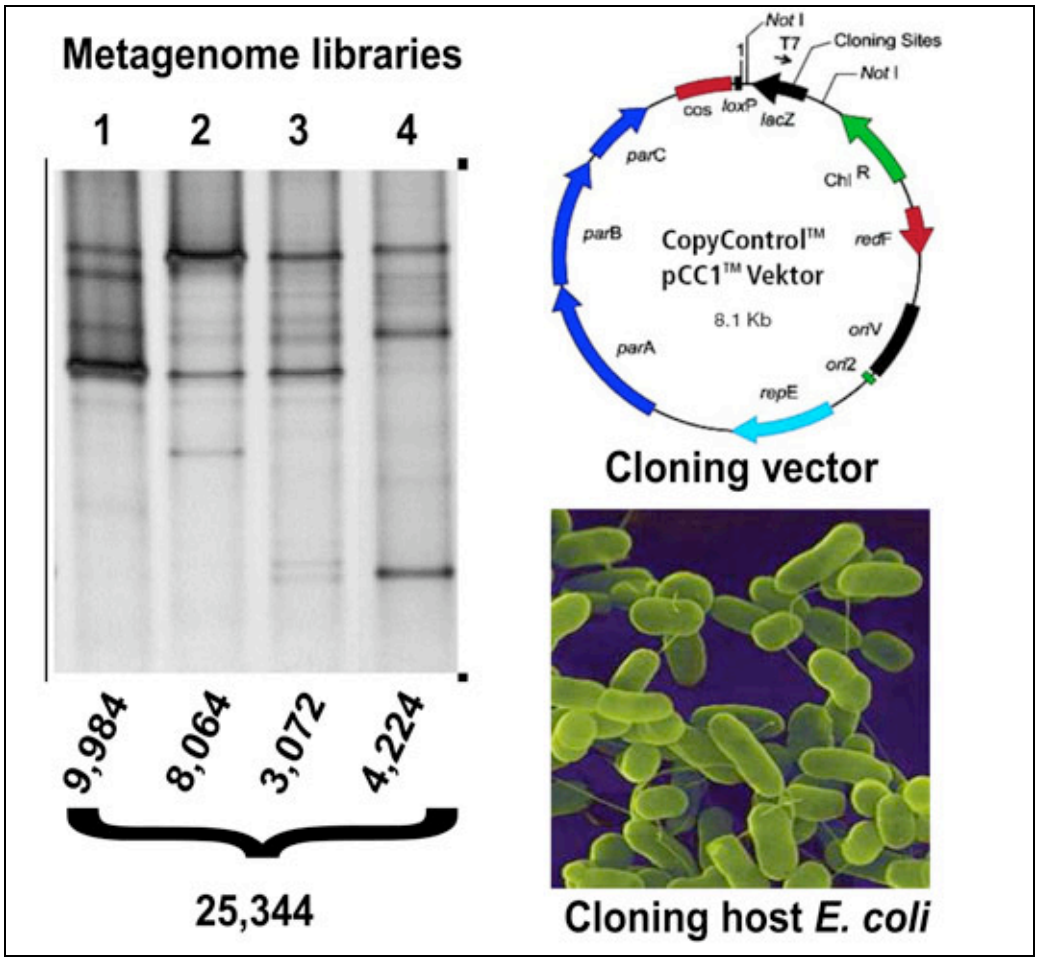

Figure 3: Schematic representation of the ileostoma effluent metagenome library construction, highlighting the diversity variation of the 4 samples used by DGGE microbiota profiling (left panel), and the numbers of fosmid clones obtained per sample. The right hand-panel shows the genetic map of the fosmid cloning vector used and a microscopic image of the *Escherichia coli* cloning host.

niche (Figure 3). Although library constructions are not required anymore for a sequence-driven metagenomics approach due to the development of cloning-independent next-generation sequencing technologies, the main reason for construction of this large-insert metagenome library was to enable the linkage between insert-sequence and functional properties per clone as they can be obtained through function-based high-throughput screening (*Handelsman*, 2004).

Various sequencing efforts were employed to investigate the diversity within the fosmid library inserts, including end-sequencing of all clones, and random sequencing of all libraries by 454-Titanium sequencing. Overall the entire sequence analyses performed generated approximately 178 Mbp of raw-sequence information. Phylogenetic positioning of all sequence reads indicated that the sequences originated from a wide variety of phylotypes, and *Clostridium* sp. *Streptococcus* sp., as

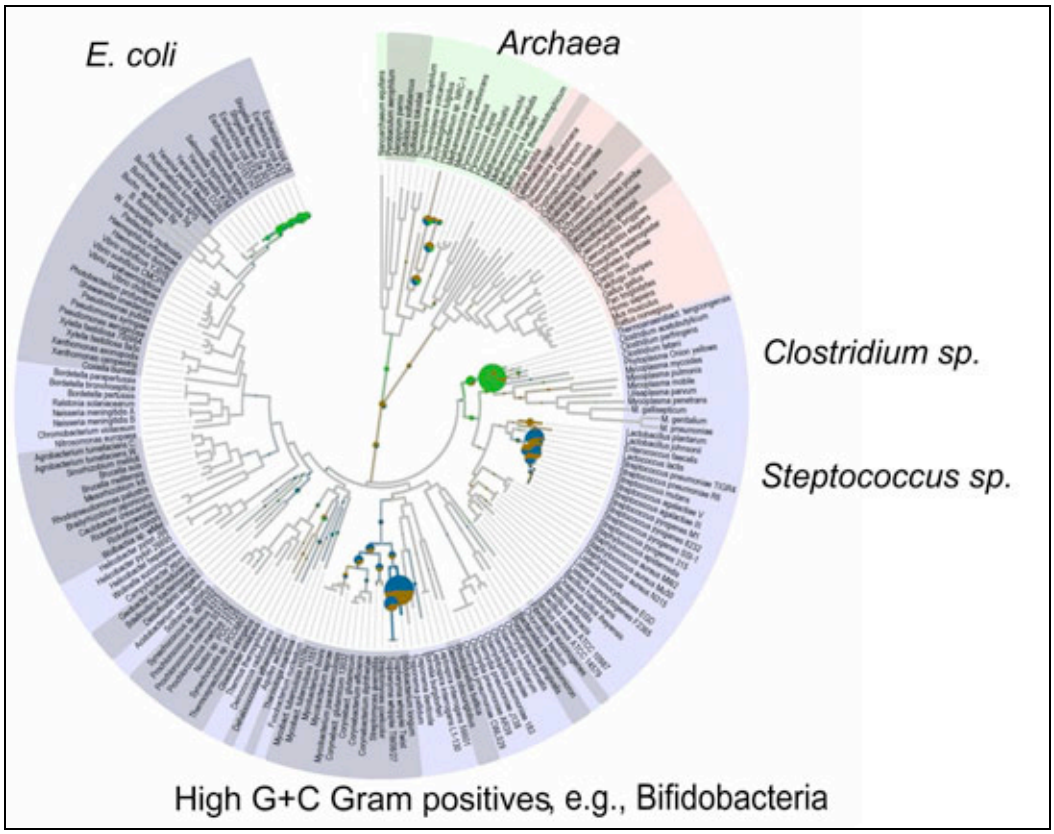

Figure 4: Phylogenetic positioning of the sequences obtained for 3 of the 4 metagenome libraries (differentially colour-coded) of the small intestine microbiota.

well as coliforms were detected among the dominant phylogenetic groups (Figure 4). Comparison of the phylogenetic profiling of the sublibraries 1, 2, and 3 revealed that all groups found in the overall analysis were found in each of the sublibraries. However, group distribution was not equal in the different sublibraries (Figure 4). The phylogenetic distribution of samples taken on the same day (2, and 3) displayed the highest similarity, and was separated from a sample that was taken a long time earlier (i.e., 1 year earlier, sample 1). The *Streptococcus* sp. and high G+C Gram-positives were overrepresented in the samples 2 and 3, while *Clostridium* sp. and coliforms were overrepresented in sample 1. This finding confirms previous observations that indicate that the microbial community in ileostoma effluent is fluctuating in time, which contrasts to the rather stable composition in the colon (*Booijink* et al., 2010). In addition, it underlines that metagenome analyses that target a single time point (i.e., the number of samples generally analyzed for intestinal metagenomics) may underestimate the overall genomic complexity encompassed within a niche, and eliminates the possibility to address the metagenome in the light of population dynamics.

Assembly of the reads per sublibrary revealed that 146 Mb of the overall sequence information could be assembled into contigs that in total en-

compass 63 Mb, leaving only 13% of the reads unassembled. This proportion of sequence reads that could be assembled is significantly higher than previously observed with faecal metagenomes (*Gill* et al., 2006; *Kurokawa* et al., 2007), containing 78 Mb and 727 Mb, respectively. This observation clearly confirms that the microbial community in the small intestine is less diverse in composition compared to that of the colon. More than 170,000 genes could be assigned to the assembled small intestine metagenome. Functional annotation of all predicted genes revealed a relatively large fraction of (conserved) hypothetical genes of unknown function (>55%). The function distribution over functional categories of all genes that could be assigned to such a category appeared to resemble the function category distribution normally observed with whole genome sequencing of individual bacteria, indicating that the cloning strategy had not introduced a too severe selective over- or under-representation of specific functional categories.

Comparative metagenomics of small and large intestine metagenome databases indicated that several pathways and functions related to carbohydrate uptake and metabolism were highly enriched in the small intestine microbiota. In contrast, membrane proteins, enzymes related to metal binding and proteins with unknown functions were enriched in the faecal microbiota. KEGG module mapping of small intestine-enriched functions underpinned a strong overrepresentation of sugar transport systems (especially PTS), and functions associated with the central carbon and energy metabolism (e.g., glycolysis and pentose phosphate pathway). For the latter category, the pathways involved in biosynthesis of sulphur containing amino acids. Similar enrichments were observed in differential analyses of categories of orthologous genes (COG) classes. The phylogenetic distribution of the genes related to central metabolic pathways was not restricted to a single phylum, but appeared to scatter across a variety of both Gram-positive and Gram-negative organisms, with slight overrepresentation of the Streptococci. This indicates that the small intestine microbiota harbours an extensive repertoire of rapid-sugar-import functions and the corresponding pathways involved in their utilization to support energy generation and growth. Next to the enrichment of these glycobiology-associated functions, a strong enrichment of the biosynthetic pathways leading to co-factor such as the vitamins cobalamin and biotin were observed. This finding may be relevant for host physiology, since biotin absorption by epithelia is known to take place in the distal region of the small intestine (*Said*, 2009), suggesting that microbes in the small intestine may significantly contribute to the human biotin supply.

Since the metagenome provides only insight into the genetic potential of the ecosystem, the actual activity pattern of the microbial community was investigated by metatranscriptomic analysis using total RNA obtained from ileostoma effluent samples. Since total bacterial RNA contains 95-99% of rRNA the sample was enriched for mRNA by selective capture methodology (Ambion microbe-Express) prior to analysis by cDNA construction and cloning. Sequencing cDNA libraries revealed that despite this enrichment still approximately 50% of all sequences were derived from rRNA, indicating that this step could be further improved. The function pattern observed in the metatranscriptome appeared to be enriched for PTS and other carbohydrate transport systems as well as glycolytic and pentose phos-

phate pathways in comparison to the metagenome of the same samples, further strengthening the importance of these functional categories in this niche. In addition, various fermentation pathways appeared to be highly expressed in the small intestine microbiota with an emphasis on those leading to production of lactate, proprionate and acetate. Taken together these findings indicate that rapid fermentation of carbohydrates is likely an important microbial characteristic that enables individual species to successfully colonize the harsh habitat of the small intestine (*Zoetendal* et al., 2010). Phylogenetic profiling of these mRNA derived sequences indicated that the majority of the transcripts detected derived from *Streptococcus* sp. and coliforms. In comparison to the metagenome database especially the fast growing facultative anaerobic organisms appeared to be prominently represented at the transcription level, suggesting high levels of gene activity in the microbes belonging to this group.

Among the dominant groups of bacteria in the small intestine that displayed the highest activity are the streptococci, which are renowned fast growing facultative anaerobic bacteria that can rapidly import and ferment relatively simple carbohydrates like mono- and disaccharides. To successfully utilize such carbohydrates in the small intestine these microbes have to compete with the host for many of these substrates. Consequently, the PTS and other transport systems as well as the downstream conversion pathways (e.g., glycolysis, pentose phosphate pathway) need to be efficient and highly expressed, which was clearly reflected by the metatranscriptome sequences. Although PTS systems of *E. coli* and relatives were also found in the metatranscriptome, their expression and the *E. coli* abundance were less compared to those of *Streptococcus* sp., suggesting that the latter bacteria most effectively fulfil the selective demands of the small intestinal habitat.

Our current model that aims to represent the small intestine microbiota focuses on the capacity for fast adaptation to the fluctuating conditions and variable nutrients as a predominant determinant for successful colonization of the small intestine habitat.

FUTURE PERSPECTIVES

This small intestine is of great importance for development and maintenance of immune homeostasis in humans. Therefore, the residing microbes may play a prominent role in tuning/modulating the local mucosal immune system, which is likely to also impact on systemic immune homeostasis. The studies presented above shed first light on the microbiota community of the human small intestine. Further studies of this dynamic microbial ecosystem are of great importance to understand its relation with host immunity. In addition, it is highly likely that dietary interventions are very effective for the modulation of the small intestine microbial community composition and function. The relative simplicity of the microbial community in the small intestine and the emerging evolutionary drivers that shape this community, enable a designer approach towards functional foods aiming to change small intestine microbiota composition and function with the ambition to modulate host immune system homeostasis. An example of such interventions may be found in the consumption of probiotics, which can drastically alter the microbial community in the small intestine temporarily and can thereby affect the

mucosal biology and immune system. Illustrative for such possibilities is the recent publication that shows that oral administration of a model probiotic organism, i.e. *Lactobacillus plantarum*, can alter mucosal gene expression patterns associated with immune regulatory networks (*van Baarlen* et al., 2009). An alternative to probiotics may lie in the composition of specific food products that stimulate/repress specific sub-groups of the endogenous small intestine microbiota aiming to alter the luminal antigen repertoire and thereby modulating local mucosal immunity. In view of the above, interventions that include administration of, or elimination of specific glyco-compounds from the diet could stimulate specific subpopulations (and/or repress other subpopulations) of the small intestine microbiota, which may be a highly effective approach to altering such luminal antigen repertoires.

Approaches towards such functional food studies that include microbiota and small-intestine mucosal analysis may employ the ileostoma subjects as a model system, since these individuals provide easy, non-invasive access to sequential samples from the small intestine of a single individual without requirements for invasive methodologies. Such studies can provide leads to dietary manipulation of the small intestine and systemic immune function by targeted alterations of microbial communities that interact with the small intestinal mucosa.

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THE MICROBIAL GUT COLONIZATION PROCESS

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SUMMARY

The composition and evolvement of early infant gut microbiota is of interest, due to developmental windows in early life that rely on stimulus from the gut. Many factors have been shown to influence the composition of early gut flora, amongst them antibiotics and caesarean delivery. Caesarean delivery is of special interest since it is associated with an aberrant gut microbiota of long lasting character and since it is increasingly more common in the western world. Furthermore, epidemiological studies indicate that caesarean delivery is associated with an increased risk of subsequent food allergy. However, the underlying mechanism is unknown. Preliminary results from the NoMic study indicate that early gut microbiota composition is diverse. It highlights the importance of studying a large number of samples from babies, and takes into account factors with gut microbiota modifying properties such as caesarean delivery, in order to increase our knowledge of early gut microbiota and its role in health and disease.

INTRODUCTION

Over the last decades an increasing amount of attention has been given to gut microbiota as we have come to understand its crucial role in a number of functions and its possible role in many diseases. The composition and evolvement of early infant gut microbiota is of special interest, due to developmental windows in early life that rely on stimulus from the gut. Specifically, early gut microbiota play a crucial role in the development of tolerance to antigens (*Sudo* et al., 1997; *Mazmanian* et al., 2005), the development of the capillary network of the gut (*Stappenbeck* et al., 2002) and in down-regulation of stress responses (*Sudo* et al., 2004). Furthermore, the colonization process during early infancy is of interest as a determinant for the subsequent adult-like microbiota (*Midtvedt*, 1994; *Hooper* et al., 1999). Knowledge of the composition of normal healthy gut microbiota and understanding the community dynamics that takes place during infancy is a prerequisite for understanding its role in disease. It may also enable us to identify early determinants for the adult ecosystem, providing an opportunity for early intervention (*Dethlefsen* et al., 2007).

The aim of this review is to discuss factors that have been shown to affect early gut microbiota while also epidemiological studies that tie such factors to child health will be presented. Finally an overview will be given of the NoMic study which, amongst others,

aims at characterizing the colonization process in babies who have not been exposed to gut modifying factors and thus may exhibit a more undisturbed colonization process.

The hygiene hypothesis proposes that the common underlying factor for allergic and other immune based diseases is lack of necessary microbial stimuli from the gut (*Guarner* et al., 2006). It is well documented in experimental studies that the commensal intestinal gut microbiota plays a crucial role in the development and maturation of the immune system. The hygiene hypothesis is indirectly supported also by a number of epidemiological studies (*Garn* and *Renz*, 2007). Interestingly, epidemiological studies also show a strong positive association between asthma and type I diabetes. Thus a country with a high prevalence of asthma has been shown to have also a high prevalence of type I diabetes (*Stene* and *Nafstad*, 2001). This supports the assumption that these two diseases may share a common underlying factor. Within an individual, however, there is some support for an opposite relation, e.g. individuals with diabetes type 1 are less likely to suffer from asthma and allergy I (*EURODIAB Substudy 2 Study Group*, 2000; *Caffarelli* et al., 2004). Thus if the hygiene hypothesis is assumed to hold true, individual susceptibility factors, such as genetic predisposition, probably play a role in whether and how lack of microbial stimulus manifests itself as disease in an individual.

Studies indicate that the composition of intestinal microbiota in babies has changed over time in the Western world (*Adlerberth* et al., 2006) and that it differs from infant gut microbiota in developing countries (*Adlerberth* et al., 1991; *Bennet* et al., 1991; *Sepp* et al., 1997). For instance, *E. coli* was a dominating early colonizer, present in most babies within three days after birth in earlier studies (*Mata* and *Urrutia*, 1971; *Gareau* et al., 1959) while more recent studies from Sweden show that a declining proportion of babies is colonized with *E. coli* (*Adlerberth* et al., 1991, 2006; *Nowrouzian* et al., 2003). Furthermore, the turnover rate of different strains is slower in western babies. Also an increase in *Staphylococcus* and a decline in *Bifidobacterium*, which dominated the microbiota of newborns in the middle of the last century, is observed (*Adlerberth* et al., 2006; *Mata* and *Wyatt*, 1971). General hygienic measures, hospital delivery, caesarean delivery and antibiotics may have played a role in these changes.

The effect of caesarean delivery on infant gut microbiota is of special interest, since the incidence of caesarean delivery is steadily increasing. For instance, in Norway only 2% of children were delivered by a caesarean section in the 1970s, 12% in the 1990s and 15% in 2001 and the incidence is still increasing (*Hager* et al., 2006). In other countries even more marked increases have taken place with half of all children now being delivered by a caesarean section (*de Moraes* and *Goldenberg*, 2001). In babies delivered by a caesarean section the input of bacteria from the maternal birth canal is missing. Thus, colonization occurs at a slower pace, partly through hospital acquired microbes. A number of studies have shown that babies delivered by a caesarean section have a delayed and different colonization, with lower colonization rates of *Bifidobacterium*, *Enterobacteriacea* and *Bacteroides* (*Biasucci* et al., 2008; *Bennet* and *Nord*, 1987; *Neut* et al., 1987; *Salminen* et al., 2004; *Grönlund* et al., 1999). Moreover, studies have indicated that the different composition may be of a long-lasting character, still evident at 7 years of age (*Salminen* et al., 2004; *Grönlund*

et al., 1999). Also of interest is a recent study, which reports an association between the mode of delivery and neonatal immune responses. The authors conclude that robust regulatory T-cell suppressive functions were observed in 59% of vaginally delivered babies compared to only 29.4% of caesarean delivered children (*Ly* et al., 2006).

An indirect way of studying whether a delayed or altered microbiota plays a role in child health is thus to study whether caesarean delivery is associated with increased risk of diabetes or allergic diseases. We have studied the associations between caesarean section and egg allergy (*Eggesbø* et al., 2003) and milk allergy/intolerance (*Eggesbø* et al., 2005), in the "Oslo Birth Cohort" which included 2803 families. Allergy to egg and milk were confirmed by a stepwise procedure which included measurement of specific IgE as well as open and double-blind placebo-controlled food challenges. Detailed information on the study sample and diagnostic procedure can be found in the published papers (*Eggesbø* et al., 2003). We did choose to study both parentally reported as well as confirmed reaction to food, since they are associated with different types of bias (misclassification and selection bias), thus consistency between the outcomes would be of importance (*Eggesbø* et al., 2003). We reported an increase of parentally reported adverse reactions to egg, fish and nuts, (but not parentally reported reactions to milk), among children delivered by a caesarean section (*Eggesbø* et al., 2003, 2005). Confirmed reactions to egg, as well as to milk, were also significantly more common among children delivered by a caesarean section. Even more interestingly, we observed that the increased risk of food allergy in caesarean delivered children was primarily confined to children born by allergic mothers (*Eggesbø* et al., 2003, 2005). These results have been published separately for egg and milk previously, and here we report them combined. Among children of non-allergic mothers the percentage of children with confirmed reactions to egg or milk was 0.6% and 0.8% among vaginally and caesarean delivered children, respectively. However, among children of allergic mothers the percentage of children that developed food allergy was 2.2% among vaginally delivered children and 6.2% among caesarean delivered children, e.g. a nearly 3-fold increased risk in food allergy among caesarean delivered children. Adjusting the results for a number of important potential confounding factors did not alter the results (*Eggesbø* et al., 2003, 2005).

Several meta analyses have recently been published which summarizes the finding of epidemiological studies on this topic. Two meta analyses have been published on food allergy and the conclusion of both is that there is support for an association between mode of delivery and food allergy (*Bager* et al., 2008; *Koplin* et al., 2008). Also a meta analysis on the association between caesarean delivery and asthma reaches the same conclusion (*Thavagnanam* et al., 2008). Finally, a meta analysis on the association between caesarean delivery and type 1 diabetes concludes that caesarean delivery is associated with a 20% increased risk of type 1 diabetes (*Cardwell* et al., 2008).

Thus epidemiological studies indicate that there is indeed an association between mode of delivery and subsequent allergy or diabetes. Although confounding by one of the many factors associated with caesarean delivery may not yet be entirely ruled out, these findings could be taken as indirect support for the "microbial deprivation hypothesis". However, if we assume that

the gut microbiota indeed plays a causal role in the association between caesarean delivery and immune related diseases, what properties of gut microbiota are driving this increased risk? Is the increased risk due to a general delay of microbial encounter, or are the aberrant "first arrivers" important, either directly or trough their influence on the colonization process? Or could a mismatch between maternal and infant gut microbiota play a role?

Despite the many unsolved questions and obvious importance of infant gut microbiota, we have limited knowledge of the overall composition of infant gut microbiota and the colonization process, since longitudinal studies targeting the dynamics occurring in this period are rare and most studies are restricted to known inhabitants of the gut (*Palmer* et al., 2007; *Adlerberth* et al., 2007; *Thompson* et al., 2008). To our knowledge only three small longitudinal studies have used an open approach based on clone libraries, which gives the possibility of discovering hitherto unknown microbial constitutions of infant gut microbiota (*Palmer* et al., 2007; *Wang* et al., 2004; *Favier* et al., 2002). A further limitation of previous studies, whether cross sectional or longitudinal, is that they are based in part on babies who have been delivered by a caesarean section, stayed at neonatal intensive care units, received early supplemental feeding or antibiotics; factors which all may have a profound disruptive effect on gut microbiota composition (*Bennet* et al., 1986; *Songjinda* et al., 2005; *El-Mohandes* et al., 1993; *Hällström* et al., 2004; *Benno* et al., 1984; *Harmsen* et al., 2000; *Yoshioka* et al., 1983; *Stark* and *Lee*, 1982; *Grönlund* et al., 2007; *Gueimonde* et al., 2007). Thus these babies may not exhibit an undisturbed colonization process.

The NoMic cohort in Norway, which consists of 524 newborns and their mothers, was established for the purpose of studying closer these issues. Faecal samples were collected from the babies at day 4, 10, 30 and at 4, 12 and 24 months. From the mothers we collected one sample after delivery. Questionnaires were filled out by the mothers at 1, 6, 12, 18 and 24 months and a follow-up is planned at 7 and 12 years. We especially ensured that detailed information on mode of delivery, indications for caesarean section, and antibiotic use during pregnancy and delivery was obtained. Microbes were identified by targeting the gene encoding ribosomal RNA (16S rRNA) (*Rudi* et al., 2007). We used an approach based on clone libraries generated from faecal samples from the study population, thus not limited to known species. We will in this study also examine microbial-dependent metabolic functions (*Midtvedt* et al., 1987; *Midtvedt* et al., 1988). More details on this cohort and the methods used will soon be published (*Eggesbø* et al., 2010).

Our first aim was to characterize the composition and natural evolvement of the gut microbiota during early infancy in Western babies who had not been exposed to a number of factors which interfere with the colonization process. We thus restricted our study samples to term babies, who had not been transferred to an intensive care unit, who were exclusively breastfed the first month of life and thereafter exclusively or partially breastfed, who were not delivered by caesarean section, and who were not exposed to antibiotics, either directly or indirectly via the mother. Interestingly, only 87 out of 524 babies fulfilled these criteria. In short, the overall composition of the infant gut microbiota among our babies corresponds well with previous studies with microbes belonging to four divisions: Firmicutes, Proteobacteria, Bac-

teroidetes and Actinobacteria (*Palmer* et al., 2007). We found that almost all newborns harboured *Staphylococcus,* γ-proteobacteria and *Bifidobacterium* in their guts four days after birth. Interestingly, even in this clearly defined sub-set of babies, we observed distinct sub-clusters of microbiota. We will further seek to identify microbial species or groups that co-evolve in a dependent manner and we will study whether any specific microbial groups can be identified as predictors for the subsequent microbiota (*Eggesbø* et al., 2010). These results will be published elsewhere.

CONCLUSION

Epidemiologic studies indicate that early infant gut microbiota composition plays a role in the development of allergic diseases. The underlying mechanism is unknown. There is a need for more information on nearly all aspects of gut colonization in early infancy. The large variation in infant gut microbiota composition highlights the need to study larger cohorts when aiming at describing gut microbiota composition and to take into account the many factors are involved in the shaping of gut microbiota.

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RESPONSE OF THE HUMAN COLONIC MICROBIOTA TO DIETARY CHANGE

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SUMMARY

Molecular methodologies allow increasingly detailed profiling of the microbial communities in terms of species composition and gene complement. This information needs to be linked to functionality, and the availability of representative cultured isolates can make an extremely important contribution by allowing functionally significant microbial markers to be monitored. A number of recent human dietary studies have shown that the species composition of the human faecal microbiota is significantly influenced by the type and quantity of non-digestible carbohydrates in the diet. Supplementation with prebiotics such as inulin not only increases bifidobacterial populations, but also those of important groups of anaerobes such as *Faecalibacterium prausnitzii* that may promote gut health. Reductions in total carbohydrate intake in weight loss diets for obese volunteers result in greatly reduced populations of the *Roseburia/E. rectale* group of butyrate producers that parallel the decrease in faecal butyrate. Most studies have so far relied on identifying phylogenetic groups that share a common function (e.g. butyrate formation) and that can be tracked using 16S rRNA-based methods. An alternative approach involves amplification of functionally relevant genes, and this has now been explored for butyrate-producing bacteria using the recently identified butyryl CoA:acetate CoA transferase gene. This type of targeted metagenomic approach allows the monitoring of previously uncultured, as well as cultured, groups of butyrate-producing bacteria. In general, there appear to be very good prospects for identifying new microbial biomarkers that are relevant to gut health.

INTRODUCTION

An unprecedented array of molecular tools is now available for analyzing the human gut microbiota. Over the past 15 years these have been based mainly on 16S rRNA sequences, allowing the diversity and abundance of different phylogenetic groupings of gut bacteria to be described in increasing detail (*Wilson* and *Blitchington*, 1996; *Suau* et al., 1999; *Hold* et al., 2002, *Eckburg* et al., 2005). Metagenomics can now provide information on the majority gene complement found in gut samples (*Gill* et al., 2006; *Kurokawa* et al.,

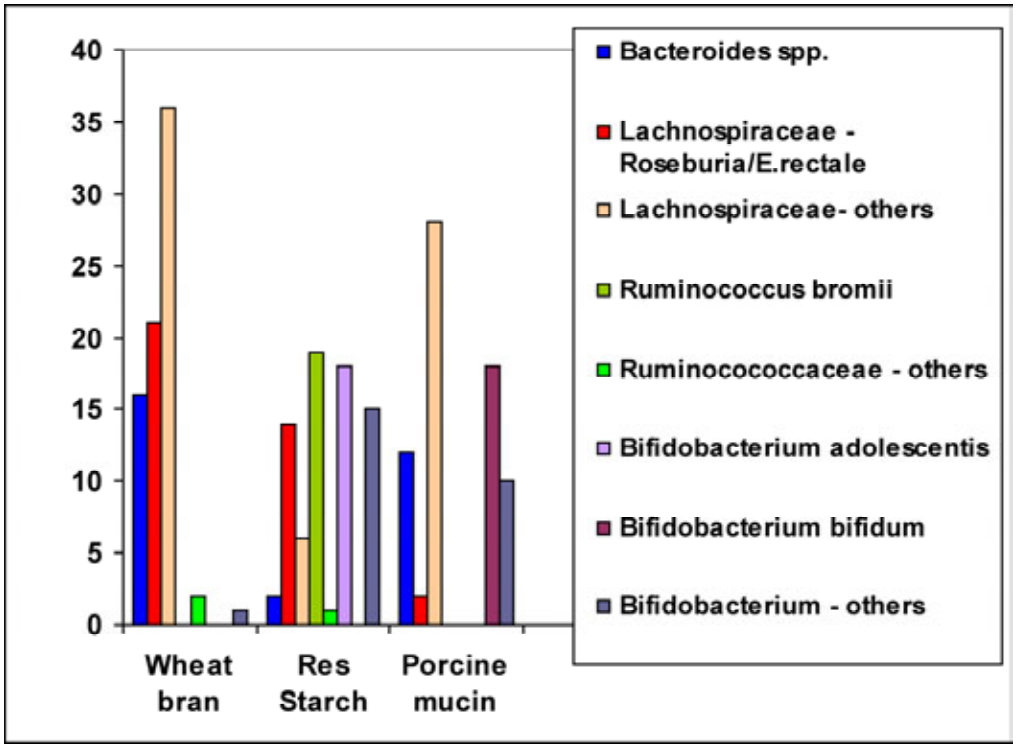

Figure 1: Selective colonization of insoluble substrates by human faecal bacteria in an *in vitro* fermentor system (*Leitch* et al., 2007). Bacterial colonization was assessed by sequencing of 16S rRNA amplicons recovered from washed residual substrate after 24 h incubation.

2007). Further technical advances make it feasible to use this information for the development of diagnostic methods, and various array-based detection approaches are being developed (*Rajilic-Stojanovic* et al., 2009).

In the rush to exploit new technologies, however, we should not overlook the importance of gaining functional information on key strains and species of gut microorganism. While acknowledging the importance of horizontal gene transfer and metabolic cross-feeding, the microbial cell remains the fundamental unit of propagation of chromosomal DNA, and of metabolism. There is great potential from combining molecular approaches with work on isolated cultures of gut bacteria through organism-based approaches (*Flint* et al., 2007).

There is increasing evidence that diet can modify the species composition of the gut microbiota. This article will consider some recent analyses of diet-induced responses as defined by molecular ecological approaches, and will then consider briefly the consequences that such shifts in the community may have upon the host.

KEY FUNCTIONAL GROUPS OF COLONIC BACTERIA

Intestinal microorganisms can be divided into functional groups based on metabolic, immunological or other criteria. As examples we will consider

here selected groups that are defined by substrate utilization and metabolic product formation.

Utilization of carbohydrate substrates: Fibre- and starch-degraders

Although many gut bacteria possess polysaccharidases, the ability to degrade plant polysaccharides, particularly when present in insoluble food particles, is less widespread (*Flint* et al., 2008). The classical approach for defining functional groups depends on the ability of isolated colonies to utilise particular substrates for growth (*Chassard* et al., 2008). A different approach recently used an *in vitro* fermentor system together with 16S rRNA sequencing to detect bacteria from human faecal samples that colonised insoluble particles of starch, wheat bran and mucin (*Leitch* et al., 2007). This revealed that colonisation was highly selective (Figure 1); in the case of starch most of the colonisers were known cultured species, but for the other two substrates many of the dominant colonisers were unknown. Such specificity is less easily detected in *in vivo* studies, since particles consist of a mixture of undigested material. Nevertheless, fractionation of faecal samples revealed that particular groups of ruminococci were significantly more abundant in the particulate than in the liquid phase (*Walker* et al., 2008). Some representatives of this group were previously isolated by *Robert* and *Bernalier-Donadille* (2003) as cellulolytic bacteria. Conversely, *Bacteroides* relatives were relatively less abundant in the particle-attached than liquid phase communities. These ecological differences are likely to reflect the different organization of polysaccharide degrading enzymes ad transport systems in different intestinal bacteria (*Flint* et al., 2008). Another novel approach is provided by stable isotope probing, which was recently used to reveal the dominant bacteria that utilise starch (*Kovacheva-Datchary* et al., 2009). Interestingly the same species were detected that attached to starch in fermentor studies (*Leitch* et al., 2007).

Product formation: Butyrate-producers

Microbially-produced butyrate is considered to be particularly important for colonic health because of its role as an energy source for the colonic epithelium and in the prevention of colorectal cancer (*Hamer* et al., 2008; *Pryde* et al., 2002). The diversity of butyrate producing bacteria in the human gastrointestinal tract has been explored by cultivation under anaerobic conditions (*Barcenilla* et al., 2000). The abundance of these cultured species has been confirmed by molecular approaches such as 16S rRNA-based fluorescent *in situ* hybridization and real time PCR, and more recently by analysis of the butyryl CoA:acetate CoA transferase gene (Figure 2; *Louis* and *Flint*, 2007; *Louis* et al., 2010). The latter technique also allows the identification of uncultured butyrate-producers, and approximately one third of the sequence groups (OTUs) detected did not correspond to cultured strains and species. These investigations have revealed three major phylogenetic groups that also display potentially significant functional differences.

Eubacterium rectale, together with four species of *Roseburia*, form one coherent group of human colonic butyrate-producers belonging to the *Lachnospiraceae*, based on 16S rRNA sequencing. They are estimated to account for 5-15% of total faecal bacteria (*Aminov* et al., 2006). Despite their extreme oxygen sensitivity, the culturability of this group appears to be high, as only one branch on the phylogenetic tree of available 16S rRNA clone li-

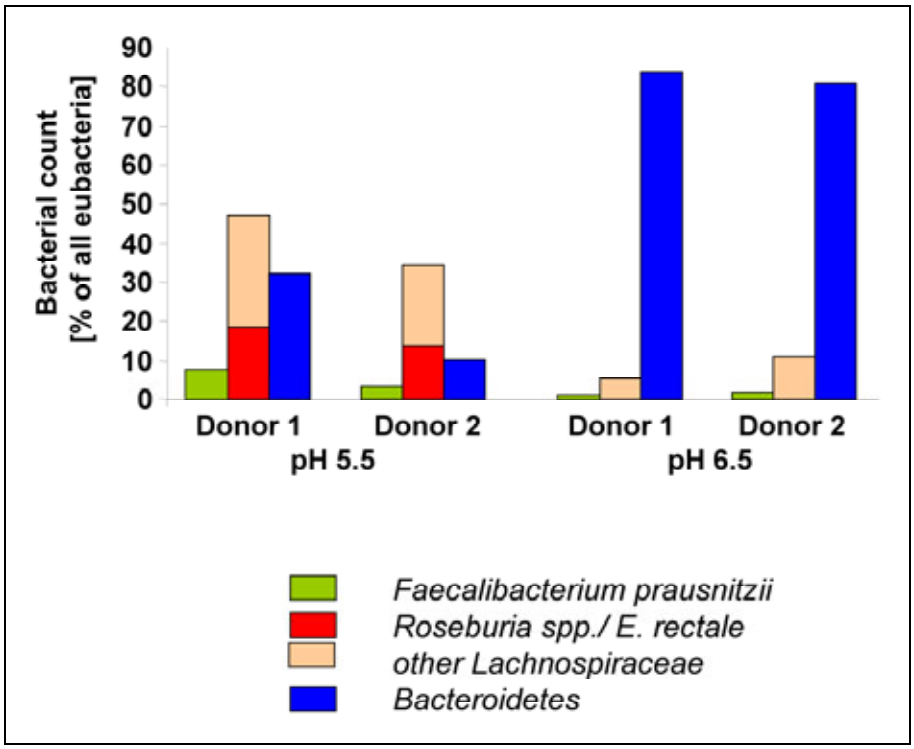

Figure 2: Abundance of presumed butyrate-producing bacteria estimated by 16S rRNA-based FISH detection (left hand columns: black = *Roseburia* + *E. rectale*, grey = *F. prausnitzii*) and by butyryl CoA: acetate CoA-transferase based quantitative PCR (right hand columns, stippled) (*Louis* and *Flint*, 2007). The samples analyzed were from an *in vitro* fermentor study on the effects of pH on human intestinal microbial communities (*Walker* et al., 2005).

brary sequences was found to lack a cultured representative (*Aminov* et al., 2006, *Flint* et al., 2007).Species of this group share several potentially significant features. They utilise acetate while forming butyrate during growth in the presence of short chain fatty acids (SCFA) and they share a unique arrangement of central butyrate pathway genes (*Louis* and *Flint*, 2009). Most are able to utilise polysaccharides, especially starch, for growth (*Ramsay* et al., 2006; *Chassard* et al., 2006). These bacteria are motile, possessing multiple flagella, and show tolerance of mildly acidic pH (*Duncan* et al., 2009).

Faecalibacterium prausnitzii represents one of the most abundant species found in human faeces, belonging to the *Ruminococcaceae*, and is also estimated to account for 5-15% of colonic bacteria. Relatively few isolates are available, but 16S rRNA sequences of the available cultured strains suggest that there is considerable diversity within the species (*Duncan* et al., 2002). Recent evidence suggests that *F. prausnitzii* may have an important role in suppressing inflammation in the gut lining (*Sokol* et al., 2008). Neither of these two prominent groups of butyrate producers shows the ability to grow with lactate as energy source. On the other hand, a third group includes species found to utilise D- and L-lactate (*E. hallii, A. caccae*) or D-lactate alone (an as yet un-named species represented by strain SS2/1) (*Duncan* et al.,

2004; *Flint* et al., 2007). This activity may be significant in stabilizing the colonic microbial community by preventing lactate accumulation and dramatic decreases in pH (*Belenguer* et al., 2007).

A recent analysis of almost 6000 16S rRNA sequences from six obese male volunteers revealed 16 dominant bacterial phylotypes that were present in all six subjects (*Walker* et al., in preparation). Eight of these were butyrate-producers: Three *F. prausnitzii,* three *Roseburia spp./E. rectale,* plus *E. hallii* and SS2/1, showing that these butyrate-producers are dominant core species within the human colonic microbiota.

IMPACT OF DIETARY CARBOHYDRATES UPON THE COLONIC MICROBIAL COMMUNITY

Profiling of the human faecal microbiota by DGGE has suggested that the bacterial species composition is relatively stable within an adult individual over time periods of a few weeks (*Zoetendal* et al., 1998). This approach provides qualitative information, however, and tends to emphasise a few very abundant bacterial ribotypes. When examined by more quantitative approaches such as FISH microscopy it is apparent that the proportions of different bacterial groups fluctuate considerably over time (*Franks* et al., 1998; *Duncan* et al., unpublished results). Such fluctuations are not surprising; different foods are consumed throughout the day, and dietary patterns typically vary through the week. Gut transit times also vary, largely in response to dietary intake. Since the gut environment and substrate availability in the large intestine must therefore change with time, the conditions for proliferation of different groups of gut bacteria will necessarily be affected.

Impact of low carbohydrate weight-loss diets on the colonic microbiota

Temporal variation clearly poses a problem for human studies aimed at understanding the impact of diet on the colonic microbiota. This problem can be minimised however if dietary intake is carefully controlled, as in some recent studies on weight loss diets in obese human subjects. *Duncan* et al. (2007) looked at the impact of two high protein weight loss diets containing reduced carbohydrates, supplied for four weeks each in a cross-over design. The low carbohydrate diet resulted in a two-fold reduction of total faecal SCFA that can be ascribed to the reduction in fibre and the virtual elimination of starch in this diet. Faecal butyrate was reduced disproportionately, around four-fold (Figure 3). Similarly *Brinkworth* et al. (2009) showed significant reductions in faecal output and in faecal butyrate and total SCFA over 8 weeks on high fat, low carbohydrate weight-loss diet. In the study by *Duncan* et al. (2007) FISH analysis of major groups within the faecal microbiota showed no significant change in the proportions of *Bacteroides* or in the overall proportion of *Clostridium*-related gut anaerobes. Two major groups of butyrate-producing *Clostridium*-related bacteria, however, behaved very differently. *F. prausnitzii* decreased only slightly as a proportion of total bacteria, but relatives of *Roseburia* and *E. rectale* decreased dramatically (Figure 4). The simplest explanation for this is that

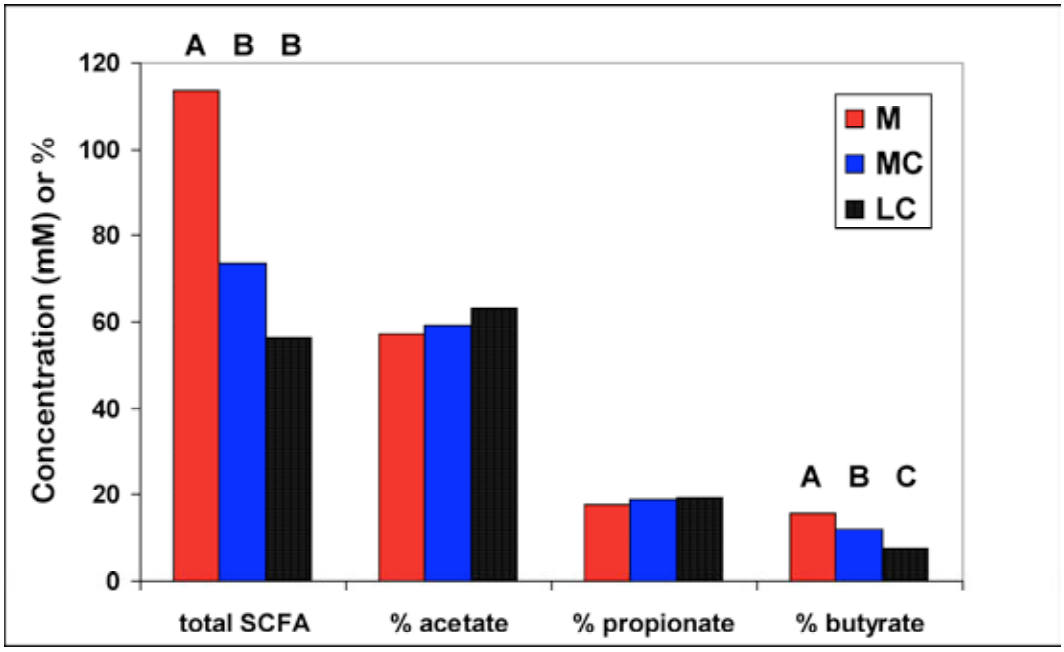

Figure 3: Impact of reduced carbohydrate weight loss diets upon faecal short chain fatty acids in a group of 17 obese male volunteers (from *Duncan* et al., 2007). Volunteers were given weight maintenance (M - mean of 399 g CHO/day for 3 days), medium carbohydrate (MC -127 g CHO/day, 4 weeks) or low carbohydrate (LC - 24 g CHO/day, 4 weeks) diets. For each metabolite shown, columns carrying different letters differed significantly ($p<0.05$).

members of the *Roseburia* group are particularly dependent on dietary resistant starch as an energy source for growth. An important compounding factor may be the effect of pH changes in response to active fermentation of dietary carbohydrates in the proximal colon, as will be discussed further below. The cross-over design of thisstudy allowed us to conclude that these changes in colonic bacteria and their metabolites were driven by diet, and not by a change in host physiology accompanying weight loss (*Duncan* et al., 2008). Both *Duncan* et al. (2007) and *Brinkworth* et al. (2009) reported reduced bifidobacterial populations on low carbohydrate, high fat diets. *Ley* et al. (2006) followed twelve obese subjects who were on either reduced fat or reduced carbohydrate weight-loss diets over 52 weeks. Their data, based on 16S rRNA clone library analysis, indicate a progressive increase on the percentage *Bacteroidetes* in the faecal microbiota with increasing weight loss. The starting percentage of *Bacteroidetes* was however far lower than has been reported in other studies on obese subjects (*Duncan* et al., 2007; *Zhang* et al., 2008; *Turnbaugh* et al., 2008).

Impact of dietary supplementation with specific carbohydrates (including prebiotics)

There is now quite extensive evidence for the modification of faecal microbiota as a result of prebiotic supplementation (e.g. *Bouhnik* et al., 2004; *Kruse* et al., 1999). Most studies have focussed on target groups such as *Bifidobacterium* and *Lactobacillus*, but some have surveyed the whole gut community. *Ramirez-Farias* et al. (2009) for example detected a significant increase in *F. prausnitzii* as well

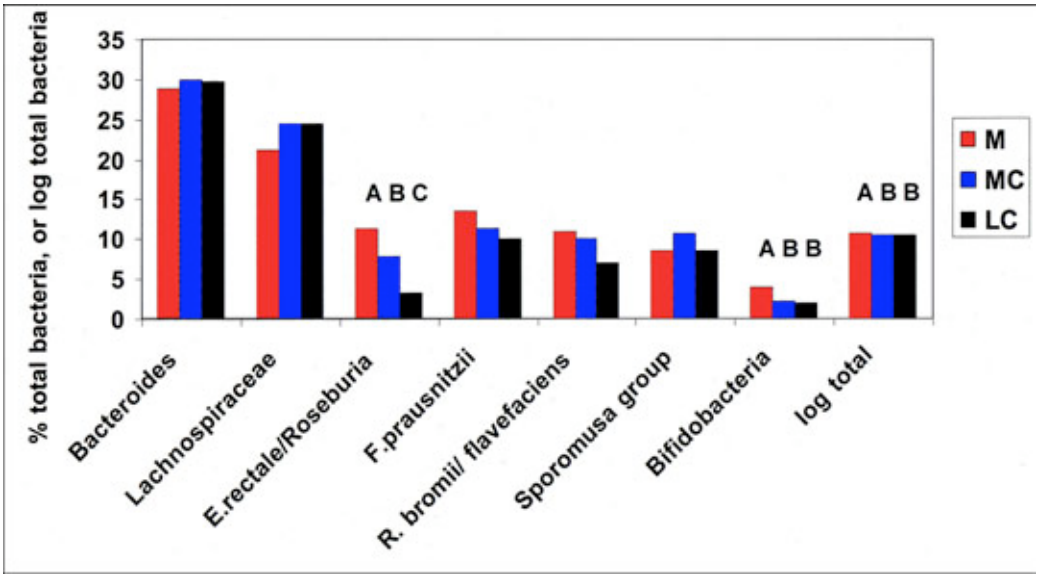

Figure 4: Impact of reduced carbohydrate weight loss diets upon bacterial populations detected in faecal samples for a group of 17 obese male volunteers (from *Duncan* et al., 2007). Diets are as described for Figure 3, and data refer to the same samples. Bacterial numbers were determined by fluorescent *in situ* hybridization (FISH) (for details of probes and conditions please see *Duncan* et al., 2007).

as in *Bifidobacterium* spp. in volunteers receiving inulin. Dietary supplementation with resistant starch (RS) also leads to significant alterations in the microbial community. Based on DGGE analysis, *Abell* et al. (2009) showed that *Ruminococcus bromii* was stimulated by RS, and this finding fits in well with recent *in vitro* work on starch utilization by faecal bacteria discussed earlier (*Leitch* et al., 2007; *Kovacheva-Datchary* et al., 2009).

Mechanisms underlying shifts in the microbial community

The most obvious explanation for a change in microbiota species composition following increased carbohydrate intake is selective stimulation of bacteria able to utilise the substrate. The overall effects are however likely to be far more complex than this. Metabolic cross-feeding, for example, will produce changes in other groups (*Belenguer* et al., 2006; *Falony* et al., 2006; *Flint* et al., 2007). In addition increased intake of fibre, resistant starch or prebiotics is likely to increase gut transit, thus influencing the gut environment and potentially altering the balance of the whole community (*Stephen* et al., 1987; *Lewis* and *Heaton*, 1997). In particular increased fermentation leads to decreased luminal pH, and this is likely to be a key factor modulating the competition and metabolism within the colonic microbiota with mildly acidic conditions shown to promote butyrate-producing bacteria and curtail *Bacteroides* spp. (*Walker* et al., 2005; *Duncan* et al., 2009; Figure 2). Very low colonic pH, reported in severe ulcerative colitis, is associated with the accumulation of lactic acid largely due to an inhibition of lactate utilization (*Belenguer* et al., 2007). In healthy subjects the pH of the proximal colon is mildly acidic whilst that of the distal region in usually closer to neutrality.

POTENTIAL CONSEQUENCES OF SHIFTS IN MICROBIAL COMMUNITY COMPOSITION

Pathogenesis

The presence of pathogenic microorganisms within the gut community presents an obvious threat of infection. This threat depends to varying degrees on the gut environment, health status, immune status and the presence of other microorganisms in the gut community. Thus certain pathogens are opportunistic, infecting only when there is an alteration in normal gut barrier function or in the resident community. For food-borne pathogens, the infectious dose is influenced by whether conditions in the gut permit survival and replication. Therefore the carriage of potentially pathogenic microorganisms and the antagonistic influence of the dominant commensal species against pathogens, are both determined by community composition. Interestingly there is evidence from a mouse model that colonisation with *Salmonella enterica* can modify the remainder of the gut community (*Stecher* et al., 2007).

Gut metabolism

Even with a gut community of constant composition it is expected that the metabolic outputs will respond to changes in the quantity and type of substrate arriving in the large intestine. The species *R. inulinivorans* for example metabolises glucose largely to butyrate, but fucose largely to propionate (*Scott* et al., 2006). Because sustained changes in substrate supply also alter community composition, as discussed above, these compositional changes will generally amplify the impact on metabolism. Thus the reduction in SCFA formation in response to decreased carbohydrate intake in obese subjects on weight loss diets (*Duncan* et al., 2007) can be attributed partly to mass action effects, but the disproportionate decrease in faecal butyrate is explained by the decreased proportion of butyrate-producing bacteria. The proportions of the major SCFA fermentation products may have an important influence upon health. Acetate may promote lipogenesis, whereas propionate is thought to help suppress cholesterol. There is increasing evidence to suggest that the proportions of the major SCFA influence gut health and metabolic health through their roles as energy sources and through their interactions with gut receptors that influence gut motility and immune responses (*Brown* et al., 2003; *Hamer* et al., 2008).

A vast array of metabolites detected in serum and urine can be ascribed to the activities of intestinal microorganisms and many of these have important health implications (*Nicolson* et al., 2005; *Holmes* et al., 2008). Some of these metabolic transformations can be attributed to minor components of the intestinal community that may show significant inter-individual variation. To give one example, *Oxalobacter formigenes* is one of the few oxalate-utilizing species in the gut community, but is found to be absent in many adults (possibly eliminated as a result of antibiotic treatment) (*Stewart* et al., 2004). These individuals show higher circulating concentrations of oxalate that may contribute to their risk of kidney stones. Interestingly, inoculation with *O. formigenes* was successful in restoring its population and reversing oxalate accumulation (*Duncan* et al., 2002). We have little idea how many significant metabolic transformations may be determined in this way by minor components of the microbiota, but they may well include metabolism of a variety of drugs and toxins.

Impact on energy supply to the host

Microbial fermentation in the large intestine is estimated to supply around 10% of the daily energy intake in humans, depending of course on the diet (*McNeil,* 1984). This energy is obtained through the uptake and metabolism of SCFA. Per mole of sugar, non-digestible carbohydrates supply less energy to the host than carbohydrates that are digested in the upper GI tract, since a significant fraction is diverted into microbial metabolism and growth (*Roberfroid,* 1999). Furthermore, many non-digestible carbohydrates such as cellulose are only partially degraded in the large intestine. This raises the interesting possibility that the extent of degradation of certain food components will be influenced by the species present in an individual's gut microbiota. *Robert* and *Bernalier-Donadille* (2003) suggested that individuals who produce methane possess different dominant species of cellulose-degrading bacteria than non-methanogenic individuals. This might be explained by the energetic advantages from the association of hydrogen-producing bacteria with methanogens and also by the likely relationship between slow transit time and methanogenesis (*El Oufir* et al., 1996). It has yet to be established however whether the extent of cellulose breakdown differs between these two groups of individuals. It has been suggested that genetically obese mice gain more energy from the diet than do lean mice, and that this reflects the higher proportion of Firmicutes than Bacteroidetes in the intestinal microbiota of the obese animals (*Turnbaugh* et al., 2006)

Impacts on the gut mucosa and the immune system

Microbial metabolites such as SCFA are known to interact with gut receptors that influence immune responses and exert anti-inflammatory effects (*Brown* et al., 2003). Furthermore there is a host of other potentially bioactive metabolites that can be formed or released from dietary components by microbial activity (*Nicolson* et al., 2005). In addition, Toll-like receptors on host cells respond to different molecular signals of microbial origin (PAMPs) such as flagellin and lipopolysaccharide (*Vijay-Kumar* et al., 2007). Changes in the distribution of such metabolic and molecular signals within the colonic community therefore have the potential to influence a wide variety of host responses. Some bacterial groups also degrade mucin or metabolise sugar residues associated with gut receptors that coat the surface of the intestinal tract (*Sonnenburg* et al., 2005). Furthermore colonisation of the gut by bacteria can influence the state of glycosylation of gut receptors (*Bry* et al., 1996).

CONCLUSIONS

Recent advances in microbial profiling and detection suggest that the human faecal microbiota can provide biomarkers for intestinal health, metabolism or dietary intake. Recent work indicated for example that the population of the *Roseburia*/*E. rectale* group was particularly dependent upon dietary non-digestible carbohydrate in the diet in obese subject on weight loss diets (*Duncan* et al., 2007). It remains to be seen however whether this relationship will hold for other subject groups and dietary regimes. One complicating factor is the extent of inter-individual variation in the gut microbiota. An-

other is the fact that most phylogenetic groups show considerable diversity and flexibility with respect to substrate utilization and this is likely to result in varied responses to different diet combinations. More promising perhaps than markers for dietary intake is the potential to correlate metabolic products or signals impacting on the host (e.g. PAMPs) with specific microbial signatures. Returning to the example above, it can be argued that populations of *Roseburia* spp. detected in faeces may provide a better measure of butyrate production in the proximal colon than can be obtained from faecal butyrate concentration. Considerably more evidence is required to test these possibilities, but such functionally-linked investigations of the gut microbiota certainly have the potential to provide valuable new biomarkers for gut health.

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HOST-MICROBE INTERACTIONS: TOWARD THE IDENTIFICATION OF MECHANISMS

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SUMMARY

The microbial community resident in the gastrointestinal tract influences the host organism in many ways. It has been recognized that the intestinal microbiota supports important physiological functions in the host and thereby contributes to disease prevention. However, the gut microbiota also plays a role in the development of certain diseases, such as inflammatory bowel disease and colorectal cancer. Many effects of the intestinal microbiota can be attributed to the immense catalytic potential of this microbial community. Beneficial activities include the activation of potentially chemopreventive substances ingested with the diet, while adverse effects are due to the formation of genotoxic or carcinogenic compounds. On the one hand intestinal bacteria contribute to the activation of lignans and isoflavones, which have been implicated in the prevention of breast and prostate cancer. On the other hand the deglycosylation of dietary arbutin by intestinal bacteria leads to the formation of mutagenic hydroquinone. The application of metagenomics revealed previously unknown correlations between the host and its gut microbiota, such as a role of the gut microbiota in obesity. The molecular mechanisms underlying such microbe-host interactions are largely obscure. However, a combination of novel tools such as the 'omics' technologies and bioinformatics, as well as classical microbiological methods and gnotobiology will help us to unravel piece by piece the molecular basis of these interactions.

INTRODUCTION

It has increasingly been recognized that the gut microbiota has a major impact on host physiology and intestinal function. However, the molecular mechanisms underlying gut microbiota-mediated effects on the host are far from being understood. Even less knowledge exists on how endogenous and exogenous factors shape the composition and function of the gut microbiota and how this in turn affects host physiology. This is largely due to the complexity of the gut microbiota and its high individual variability. Recent advances in high throughput sequencing, transcriptome and microbiome analysis as well as bioinformatics enabled the recognition of previously unknown functions of the gut microbiota and provided new insights into how intestinal bacteria exert their effect on the host. However, in spite of all of these great advances it

also has become clear that we are still far away from understanding the molecular basis of the multi-faceted host-microbe interactions. We therefore need to keep on exploring this fascinating ecosystem with a wide range of experimental approaches.

REVIEW AND DISCUSSION

Roles of intestinal bacteria in disease

Intestinal bacteria have been implicated in a number of diseases, including inflammatory bowel disease, colon cancer and, more recently, the metabolic syndrome. Inflammation of the bowel as observed in Crohn's disease and ulcerative colitis, becomes manifest in the susceptible host whose immune system has not acquired tolerance toward the antigens of commensal gut bacteria (*Sartor*, 2006). Whether, and to which extent, specific organisms play a role in the manifestation of this disease is still unclear. In any case, inflammatory bowel disease exemplifies the intricate relationship between the host and its microbiota, in particular its interaction with the host immune system.

Gut microbiota and inflammation

We studied the intestinal microbiota composition in the IL-10-deficient mouse (IL-$10^{-/-}$), a widely accepted model for chronic colitis. Molecular microbial population analysis revealed a decreased microbial diversity in the inflamed gut, with *Escherichia coli* and *Blautia producta* becoming the dominant bacterial species (*Wohlgemuth* et al., 2009). Phylogenetic grouping revealed that all mice (IL-$10^{-/-}$ and wild-type) were colonized by one single *E. coli* strain with the serotype O7:H7:K1. We detected a high number of virulence- and fitness-associated genes in this strain's genome, which possibly are involved in the organism's adaptation to the murine intestine. When this strain was introduced into germ-free mice together with two other *E. coli* strains, the isolate overgrew its competitors. However, we found no evidence that the strain causes gut inflammation in conventional animals. We therefore conclude that the observed growth stimulation of *E. coli* in gut inflammation is rather a consequence than cause of the disease.

Formation of genotoxic compounds

The role of the intestinal microbiota in colon cancer development is more related to its immense catalytic potential which has been compared to that of the liver. Intestinal bacteria convert both endogenous and exogenous substrates. The bacterial conversion in the intestine of some of these substrates may lead to the formation of genotoxic compounds. Secondary amines and phenols are typical products of the bacterial breakdown of proteins in the distal colon (*Macfarlane* et al., 1988). In conjunction with nitrite they may lead to the formation of nitrosamines and *p*-nitrosophenol (*Kikugawa and Kato*, 1988), both of which are highly carcinogenic.

Genotoxic compounds may also arise from the bacterial conversion of non-nutritive dietary components, such as arbutin that is found in pears, coffee and wheat. We investigated the deglycosylation of arbutin by intestinal bacteria, which convert this compound to hydroquinone, a mutagenic and therefore potentially carcinogenic substance

(*Blaut* et al., 2006). Furthermore the conversion of neoglucobrassicin, a glucosinolate found in vegetables such as broccoli and pak choi, leads to the formation of DNA adducts in the mucosa of the upper part of the intestinal tract when consumed raw, but preferentially in the colonic mucosa when consumed after cooking. In the former case plant-derived myrosinase catalyzes the cleavage of the compound while in the latter case preferentially intestinal bacteria are assumed to catalyze this activation because no DNA adducts are found in germ-free animals. These examples show that the intestinal microbiota catalyzes a wide range of reactions that may have adverse health effects on the host.

Roles of intestinal bacteria in health

Contribution to intestinal barrier

Intestinal bacteria contribute to the intestinal epithelial barrier, which prevents the translocation of undesirable components from the gut lumen into the underlying host tissues and the circulation (*Berg*, 1996). This barrier effect is of utmost importance as it largely prevents the invasion of intestinal tissue by pathogens and ensuing disease. Competitive exclusion of invaders impedes their establishment and persistence in the ecosystem unless they are capable of successfully competing for nutrients and binding sites. The intestinal microbiota also fortifies the intestinal barrier by keeping the mucosal immune defence in a state of alertness. Various host cells possess so-called pattern recognition receptors (*Cario*, 2005) that sense and identify bacterial cells based on their characteristic cell components, and, following the detection of pathogens they trigger an immune response.

Formation of short-chain fatty acids

Another important function of the gut microbiota lies in its ability to process non-digestible dietary as well as endogenous components. Dietary fibre, which includes a wide range of oligomeric and polymeric carbohydrates, escapes digestion in the small intestine and, upon reaching the colon, undergoes fermentation by intestinal bacteria. Dietary fibre not only is the main source of energy for intestinal bacteria but also leads to the main products of bacterial fermentation, preferentially the short chain fatty acids acetate, propionate and butyrate (*Mortensen* and *Clausen*, 1996). Besides providing energy to the host it has been recognized that short chain fatty acids are important for the maintenance of a healthy colonic mucosa. In particular butyrate has been implicated in the prevention of ulcerative colitis and colon cancer owing to its effect on epithelial cell proliferation and cell differentiation (*Andoh* et al., 2003).

Bio-activation of dietary components

Intestinal bacteria also play a major role in the activation of non-nutritive dietary components such as polyphenols. These stem from dietary plants and include tannins, lignans and flavonoids. The latter two groups are in the focus of intense research because both epidemiological and experimental data indicate that they may play an important role in disease prevention. For example, isoflavones and lignans have been implicated in the prevention of breast and prostate cancer, osteoporosis, menopausal symptoms, and cardiovascular diseases. Following their oral intake, polyphenols may undergo trans-

formation by intestinal bacteria which in turn influences both their bioavailability and bioactivity in the human intestinal tract.

The conversion by intestinal bacteria of non-nutritive dietary components, such as polyphenols, does not appear to be essential for the gut microbial community as a whole. This may be deduced from the fact that the conversion of some of these substances is not observed in all individuals but only in a subpopulation. For example, only 30-50% of human subjects excrete equol in their urine in response to the consumption of soy, which is a major source of daidzein (*Atkinson* et al., 2005). Intestinal bacteria convert the isoflavone daidzein and the lignan secoisolariciresinol-diglucoside (SDG) to equol and enterolactone, respectively. The latter have been implicated in the prevention of sex hormone-related cancers, osteoporosis and the alleviation of menopausal symptoms (*Thompson* et al., 2005). Isoflavones and their metabolites may bind to oestrogen receptors and exert agonistic or antagonistic effects (*Scalbert* et al., 2005).

Microbial conversion of lignans

Lignans undergo activation by intestinal bacteria to adopt estrogenic and anti-oxidant activities. We recently isolated and identified human intestinal bacteria involved in lignan activation and characterized all steps leading from the plant lignan SDG to enterodiol (ED) and enterolactone (EL) (*Clavel* et al., 2005, 2006a,b). We described two of these isolates as new species: *Clostridium saccharogumia* and *Lactonifactor longoviformis*, with the latter representing a new genus (*Clavel* et al., 2007). The ability of faecal bacteria to convert SDG to the oestrogen-like metabolites ED and EL was found to be widely distributed among humans. Women tended to show higher concentrations of both ED- and EL-producing organisms.

Microbial conversion of isoflavones

To study the conversion of isoflavones in more detail, we isolated and characterized two bacterial strains from the mouse intestine and from human faeces, respectively (*Matthies* et al., 2008, 2009). The strains converted daidzein via dihydrodaidzein to equol. Likewise, the new isolates formed 5-hydroxy-equol from genistein via dihydrogenistein. The conversion of daidzein and genistein depended on the pre-incubation with these isoflavones, indicating that the corresponding enzymes are inducible. Both isolates are new species belonging to the *Coriobacteriaceae*.

These few examples highlight the profound impact of the intestinal microbiota on the host metabolism and how nutrition affects this correlation. However, although we are aware that the intestinal microbiota affects the host in many ways it is very likely that only a small proportion of the effects conferred by gut bacteria on the host have been recognized. We know even less on how these effects are brought about. There are numerous questions that have not yet been answered. These include: Which nutritional, environmental and host factors govern the development of the microbial community in the newborn? What explains the immense microbial diversity of the gut microbiota at the species and strain level in spite of similar key functions? How do host and nutrition factors affect the composition and functional activity of the gut microbiota?

The metagenomic approach

The advent of the 'omics' technologies and the ease and speed of high-throughput sequencing has opened new opportunities for investigating the gut microbiota and its interactions with the host. Sequencing of all genes present in the genomes of all members of the gut microbial community in different individuals is part of the Human Microbiome Project (*Turnbaugh* et al., 2007). This project preferentially aims to determine whether individuals share a core human microbiome and whether changes in this microbiome can be correlated with health and disease. Metagenomics is thought to provide relatively unbiased information about the community structure and its functional potential (*Hugenholtz* and *Tyson*, 2008). The application of a metagenomic approach to the human gut microbiota revealed previously unknown correlations. For example, the observation that the association of germ-free mice with a microbiota obtained from obese mice results in a greater increase in total body fat than with a microbiota from lean mice prompted an investigation using a metagenomic approach. As a result, the gut microbiota was identified as a factor that may contribute to the pathophysiology of obesity. Taxonomic analyses in the obese host revealed a higher proportion of intestinal bacterial cells belonging to the Firmicutes and Actinobacteria than to the Bacteriodetes. This has been demonstrated for both mice and humans (*Turnbaugh* et al., 2006, 2009). These taxonomic differences in the microbiome of the obese host were accompanied by an enrichment of genes encoding enzymes involved in the breakdown of dietary polysaccharides that escape digestion in the small intestine and in enzymes involved in lipid and amino acid metabolism. These genes were proposed to comprise a set of microbial biomarkers characteristic of the gut microbiome of the obese host.

Although only few laboratories have the equipment and the powerful bioinformatics at their disposal to analyze the huge amount of data being produced in the course of such studies, it has to be acknowledged that the metagenomic approach offers the unique opportunity to discover new correlations between host and gut microbiota. However, usually the metagenomic approach does not reveal the molecular mechanisms underlying an observed correlation between a given physiological or pathophysiological status of the host and an enrichment of certain genes, which reflect the microbiota composition and the metabolic and functional potential of the microbial community. Another drawback of metagenomics lies in the fact that the gap between characterized and hypothetical proteins is getting bigger as more and more sequence data become available (*Hugenholtz* and *Tyson*, 2008).

The study of host-microbe interactions

'Omics' technologies in conjunction with the use of animal models with a defined microbial status have lent momentum to the study of host-microbiota interactions and their underlying molecular mechanisms. Various publications demonstrate the power of this approach. For example, it has been shown that the gut microbial community shapes the intestinal environment: In mice, intestinal bacteria are essential for the continuation of a differentiation

program which is initiated after birth and leads to the fucosylation of the small intestinal epithelium; one intestinal species, namely *Bacteroides thetaiotaomicron*, was sufficient to induce the expression of fucosyltransferase in small epithelial cells (*Bry* et al., 1996). Other recent investigations indicate that the gut microbial community affects both nutrient harvest and energy metabolism of the host (*Backhed* et al., 2004, 2005, 2007). The influence of the gut microbiota on the host energy metabolism is considered to be of particular relevance in view of the epidemic increase in obesity.

The availability of the complete genomic sequence of man and mouse prompted investigations into host gene expression in different intestinal tissues in response to bacterial colonization of the gastrointestinal tract. Germ-free mice were mono-associated with *Bacteroides thetaiotaomicron* (*Hooper* et al., 2001), a numerically dominant member of the intestinal microbiota, which is known for its versatility in the use of complex carbohydrates as energy substrates. A large variety of genes involved in nutrient absorption, detoxification of xenobiotics, intestinal maturation, mucosal barrier function and innate immunity were shown to undergo changes in their expression in response to the association with this organism. A wide range of genes involved in intestinal maturation, mucosal barrier function, nutrient uptake and conversion of xenobiotics, were increased by more than two-fold in response to the association with *B. thetaiotaomicron*. The increased expression of the genes encoding Na^+/glucose co-transporter (SGLT1), pancreatic lipase-related protein (PLRP-2), co-lipase, liver fatty acid binding protein (L-FABP) and apolipoprotein A-IV indicate that *B. thetaiotaomicron* improves nutrient absorption. This is in line with the observation that conventional rodents gain 40% more body fat than their germ-free counterparts in spite of a lower consumption of a standard rodent chow diet (*Backhed* et al., 2004).

The genomes of a number of bacterial species relevant for the human intestinal tract have been sequenced. Analysis of the *B. thetaiotaomicron* genome indicated that this organism has an arsenal of enzymes devoted to the utilization of a large variety of carbohydrates (*Xu* et al., 2003). A comparison of gene-expression profiles of *B. thetaiotaomicron* from mice mono-associated with this organism and fed either a fibre-free diet or a diet containing fermentable fibre, indicated that the bacteria from the mice fed the former diet primarily expressed genes involved in the breakdown of host-derived substrates such as mucins and chondroitin sulphate (*Sonnenburg* et al., 2005). In contrast, the bacteria obtained from the mice fed the latter diet expressed genes involved in the degradation of fermentable dietary fibre such as resistant starch and pectin. These data indicate that host and dietary factors influence bacterial gene expression in such a way that intestinal bacteria are capable of optimally adapting to a given metabolic situation.

The role of intestinal bacteria in fortifying the mucosal barrier and thereby improving colonization resistance has many facets, one of which has been investigated in more detail in mice mono-associated with *B. thetaoitaomicron*. Colonization of germ-free mice with this organism, but also lipopolysaccharides from *Salmonella* induce the expression of Angiogenin-4 (Ang4), which originally was assumed to play a role in angiogenesis but later on Ang4 was shown to be a cryptdin, an antimicrobial defensin produced by Paneth cells and secreted into the gut

lumen (*Hooper* et al., 2003). Ang4 is effective against a number of Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes* and *Enterococcus faecalis*. This mechanism contributes to the maintenance of epithelial integrity and helps to protect the host from detrimental environmental effects. Once the barrier becomes disrupted, bacterial and food antigens have access to the sub-mucosa. Here they may induce an inflammatory response which, if uncontrolled, may lead to inflammatory bowel diseases.

These examples show that for mechanistic studies it is necessary to dissect the complex interactions between the host and its microbiota. This can be accomplished by applying a reductionistic approach, which helps to minimize the number of confounding parameters. In addition it will remain necessary to investigate new bacterial activities which may be considered minor but which may have important consequences for the host.

Bacterial response to the host environment

The effects of the gut microbiota on the host have been studied extensively. In contrast, the microbial response to host factors has hardly been considered although this response may have implications for the host. We took advantage of a reductionistic animal model for investigating how an intestinal bacterium adjusts its physiology to the specific conditions in the gastrointestinal tract. We associated germ-free mice with commensal *Escherichia coli* as a simplified model of host-bacteria interactions. We analyzed the bacterial adaptation to the gut environment by a proteomic approach using two-dimensional gel electrophoresis followed by electron-spray ionization-tandem mass spectrometry (*Alpert* et al., 2005). We characterized 60 arbitrarily chosen protein spots and identified 50 unique bacterial proteins. Their ascribed functions suggest that the host-associated bacteria adapt their metabolism to the simultaneous use of a wide spectrum of substrates available in the intestinal tract. This differs completely from the situation in nutrient-rich media where the metabolism of *E. coli* is strictly regulated so that preferably only one substrate is utilized at a time. We detected ten proteins with unknown or poorly characterized physiological functions and three proteins whose existence so far had been inferred from predictions only. We assume that some of these proteins play a role in the bacterial adaptation to the host environment (*Alpert* et al., 2009). We are now in the process of producing *E. coli* strains in which the genes of interest have been knocked out in order to study the effect of these gene knockouts on the cells' ability to colonize germ-free mice and to compete with the corresponding wild type strain.

CONCLUSIONS

Interest in the intestinal microbiota has been largely triggered by the awareness that this microbial community affects the health status of the host organism. Technological progress enabled the development of novel experimental approaches, such as the metagenomic approach, which revealed correlations between host health and the microbiota. Observations include the enrichment or

the depletion of members of the bacterial community or sets of genes. However, which mechanisms underlie the observed effects has largely remained obscure. Similarly, there is little knowledge on how host and nutrition factors shape the intestinal microbiota. To get mechanistic insights into these processes it is mandatory to improve the knowledge base by combining a whole range of methods and tools. Not only will it be necessary to keep on isolating new intestinal bacteria with relevant activities, but we also need to diminish the proportion of genes with unknown functions. All of this involves classical microbiology and biochemistry. This in conjunction with the use of gnotobiotic and knockout animals will bring us closer to a better understanding of the mechanisms underlying host-microbe interactions.

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SEGMENTED FILAMENTOUS BACTERIA: KEY DRIVERS OF THE MUCOSAL IMMUNE MATURATION

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SUMMARY

Association of germ-free mice with a complete intestinal microbiota promotes the development of the mucosal immune system. *Clostridium*-related, segmented filamentous bacteria (SFB) are non-cultivable, commensal bacteria that strongly adhere to the epithelial cells of the ileum and of Peyer's patches at which time they competitively inhibit adhering pathogens. Colonizing SFB induce IgA-producing cells in the lamina propria of the small intestine and αβ-T-cell-receptor bearing intra-epithelial lymphocytes. As a consequence of this immuno-activation, adherence of SFB declines in time. The level of immune activation exceeds that of other non-pathogenic bacterial strains or non-host microbiota. In conclusion, SFB are key players in the maturation of the murine immune system of the gut. In this review, taxonomic data, interactions with the host and its immune system, and factors influencing colonization are highlighted.

INTRODUCTION

The bacterial community in the mammalian gastrointestinal tract comprises an estimated several hundreds of different species and as many as 10^{11}-10^{12} bacteria per gram of content in the colon. Many species are difficult or as yet impossible to culture, and it is only recently that techniques are available to describe the complex intestinal microbial ecosystem by molecular techniques without the need to culture bacteria (*Vaughan* et al., 2000; *Dethlefsen* et al., 2008).

Bacterial colonization in the small intestine is far lower than in the colon, and in major part concentrated to the terminal part the ileum. So-called Segmented Filamentous Bacteria (SFB) belong to the most remarkable bacterial species in the ileum. These bacteria are defined on the basis of their morphology and habitat: Long chains ('filaments') of spore-forming bacterial cells ('segments') that are attached with one end of the filament to the intestinal wall (Figure 1). In mammals, they preferentially adhere to the epithelial cells of the ileum and of Peyer's patches, small lymphoid organs involved in antigen sampling from the intestinal lumen. Despite intense association with the gut wall, SFB generally do not possess pathogenic characteristics.

Because of the intimate relationship of SFB with the host and non-pathogenic nature, it has been speculated for years that they might increase host resistance against intestinal infections (*Glick* et al., 1978; *Porvaznik* et al.,

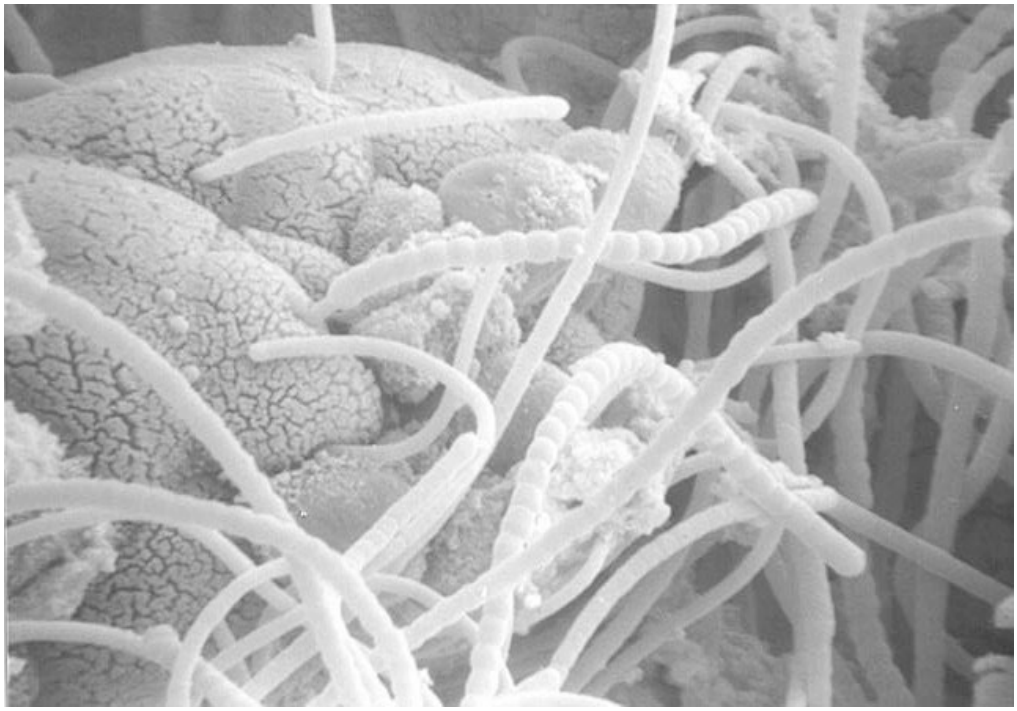

Figure 1: Scanning electron microscopic image of segmented filamentous bacteria in mice. Filaments are attached to epithelial cells without any signs of inflammation. Two morphotypes can be distinguished: smooth filaments and filaments with a beaded appearance.

1979). Several studies have demonstrated an important role for these bacteria in the maturation of the mucosal immune system. SFB are recognized to strongly stimulate IgA production (*Klaasen* et al., 1993a; *Snel* et al., 1997), and intra-epithelial lymphocytes (*Umesaki* et al., 1995) in the small intestine when germ-free mice become colonized with these bacteria.

PHYLOGENY AND TAXONOMIC STATUS

Intestinal spore-forming bacteria with a segmented filamentous appearance that attach to the intestinal wall were originally described in mice (*Hampton* and *Rosario*, 1965) and chickens (*Fuller* and *Turvey*, 1971), but have now been found in a wide range of animals (Table 1). Although never cultured *in vitro*, monocultures of SFB have been established by using intestinal homogenates of donor mice that were treated with filtered ethanol, diluted and administered intra-ileally to recipient germ-free mice (*Klaasen* et al., 1991a; *Umesaki* et al., 1995). These cultures have been used to determine the 16S ribosomal RNA sequence of SFB and for subsequent phylogenetic analysis (*Snel* et al., 1994).

Analysis of the available ribosomal RNA sequences from SFB in mice, rats and chicken revealed that these bacteria form a natural group, which is peripherally related to the genus *Clostridium* sensu stricto (group I *Clostridium*) (*Snel* et al., 1995). Later de

Table 1: Overview of animal species in which intestinal spore-forming bacteria with a segmented filamentous appearance that attach to the intestinal wall have been observed

Species	Site	Adhesion and morphology revealed by microscopy	16S rRNA sequence data described
Mice	ileum	fluorescent *in situ* hybridisation using SFB- specific probe (*Snel* et al., 1994); scanning electron microscopy (*Hampton* and *Rosario*, 1965; *Davis* and *Savage*, 1974)	*Snel* et al., 1994, 1995; *Imaoka* et al., 1997
Rats	ileum	(*Tannock* et al., 1984)	*Snel* et al., 1995; *Imaoka* et al., 1997
Chicken	ileum, proximal caecum	(*Fuller* and *Turvey*, 1971; *Pearson* et al., 1982; *Angel* et al., 1990; *Allen*, 1992)	*Snel* et al., 1995; *Gong* et al., 2007
Dog	ileum	scanning electron microscopy (*Davis* et al., 1977; *Hoskins* et al., 1982; *Klaasen* et al., 1993b)	
Cat	ileum	scanning electron microscopy (*Gregory* et al., 1985)	
Rabbit	ileum	scanning electron microscopy (*Heczko* et al., 2000)	
Sheep	ileum	scanning electron microscopy (*Gregory* et al., 1985)	
Rainbow trout	distal intestine	fluorescent *in situ* hybridisation using SFB- specific probe (*Urdaci* et al., 2001)	*Urdaci* et al., 2001
Carp	small intestine	light microscopy (*Klaasen* et al., 1993b)	
Horse	ileum	scanning electron microscopy (*Lowden* and *Heath*, 1995)	
Pig	ileum	*Sanford*, 1991	
Crab-eating monkey	ileum and caecum	light microscopy (*Klaasen* et al., 1993b)	*Imaoka* et al., 1997
Rhesus monkey	ileum	light microscopy (*Klaasen* et al., 1993b)	
Vervet monkey	ileum	scanning electron microscopy (*Bruorton* et al., 1991)	
Humans	faeces, ileum	light microscopy (*Klaasen* et al., 1993b)	hybridization with SFB-specific probe (*Child* et al., 2006)

rived sequences from rainbow trout (*Urdaci* et al., 2001) and crab-eating monkeys (*Imaoka* et al., 1997) fall in the same group. Since these bacteria cannot be cultured, they are not submitted to any culture collection, and can only be described on the basis of their phylogeny and morphology. Therefore, as a provisional genus name, "*Candidatus* Arthromitus" was proposed (*Snel* et al., 1995).

SFB are host specific: Ileal bacterial preparations containing SFB did only lead to outgrowth in ex-germ-free mice and rats when they where derived from the same species (*Tannock* et al.,

1984). Also faecal preparations from SFB-mono-associated mice did rarely lead to attachment to the ileum of rats, although colonization of the caecal content was observed (unpublished data). Because of this host specificity, species names "*Candidatus* Arthromitus muris", "*Candidatus* Arthromitus ratti" and "*Candidatus* Arthromitus galli" were suggested for SFB in mice, rats and chickens respectively (*Snel* et al., 1995)

A few studies have described SFB-like microorganisms in invertebrate species, such as the hindgut of termites (*Margulis* et al., 1990) and cockroaches (*Bracke* et al., 1979). Nevertheless, later studies revealed that the segmented, filamentous microorganisms in these species were in fact *Bacillus cereus* (*Margulis* et al., 1998). The authors concluded that *B. cereus* and its close relatives, easily isolated from soil and grown on nutrient agar, enjoy filamentous growth in moist, nutrient-rich intestines of healthy arthropods and similar habitats. This illustrates that firm conclusions on the presence of SFB can only be drawn when genetic information such as ribosomal RNA sequence data are available together with microscopic evaluation.

The wide range of vertebrate animal species in which SFB is found (Table 1), including birds, fish and mammals, suggest that bacteria from this group are present in humans too. Although an SFB-specific 16S ribosomal RNA targeted probe SFB did hybridize with bacteria in a continuous culture model inoculated with human faeces (*Child* et al., 2006), and long filamentous organisms are observed by light microscopy in human ileal biopsies (*Klaasen* et al., 1993b), conclusive evidence that SFB form a natural part of the intestinal microbiota in humans is still lacking.

MICROSCOPIC OBSERVATIONS

As being related to *Clostridium*, SFB are endospore-forming microorganisms (*Chase* and *Erlandsen*, 1976). As expected for sporeformers, SFB are resistant to treatment with chloroform or ethanol, but surprisingly sensitive to temperatures above 55°C. Microscopic analysis of SFB revealed that its spores contain two daughter cells instead of one as observed in most spore-forming species. Multiple daughter cells in single spores have also been reported in *Metabacterium* and *Epulopiscium* species (*Angert* et al., 1996). Although *Metabacterium polyspora* is a common member in the guinea pig caecal microbiota, it is like *Epulopiscium* spec. not directly related to SFB.

Filaments of SFB in mice are either smooth or have a beaded appearance. One end of the filament is attached to the epithelial wall with a special structure, described as holdfast (Figure 2). A life cycle is proposed in which spores release holdfasts that subsequently adhere to the ileum and grow out to filaments of 50-1000 μm (*Klaasen* et al., 1992a).

Several electron microscopic studies in mice describe Gram-positive rod-shaped bacteria adhering to the SFB filaments in mice (*Koopman* et al., 1987; *Klaasen* et al., 1992a). The exact nature of these bacteria is unknown, although it is speculated that they may be *Lactobacillus* species (*Koopman* et al., 1987). Similar sub-colonization of SFB by rod-shaped bacteria is found in chickens (unpublished data).

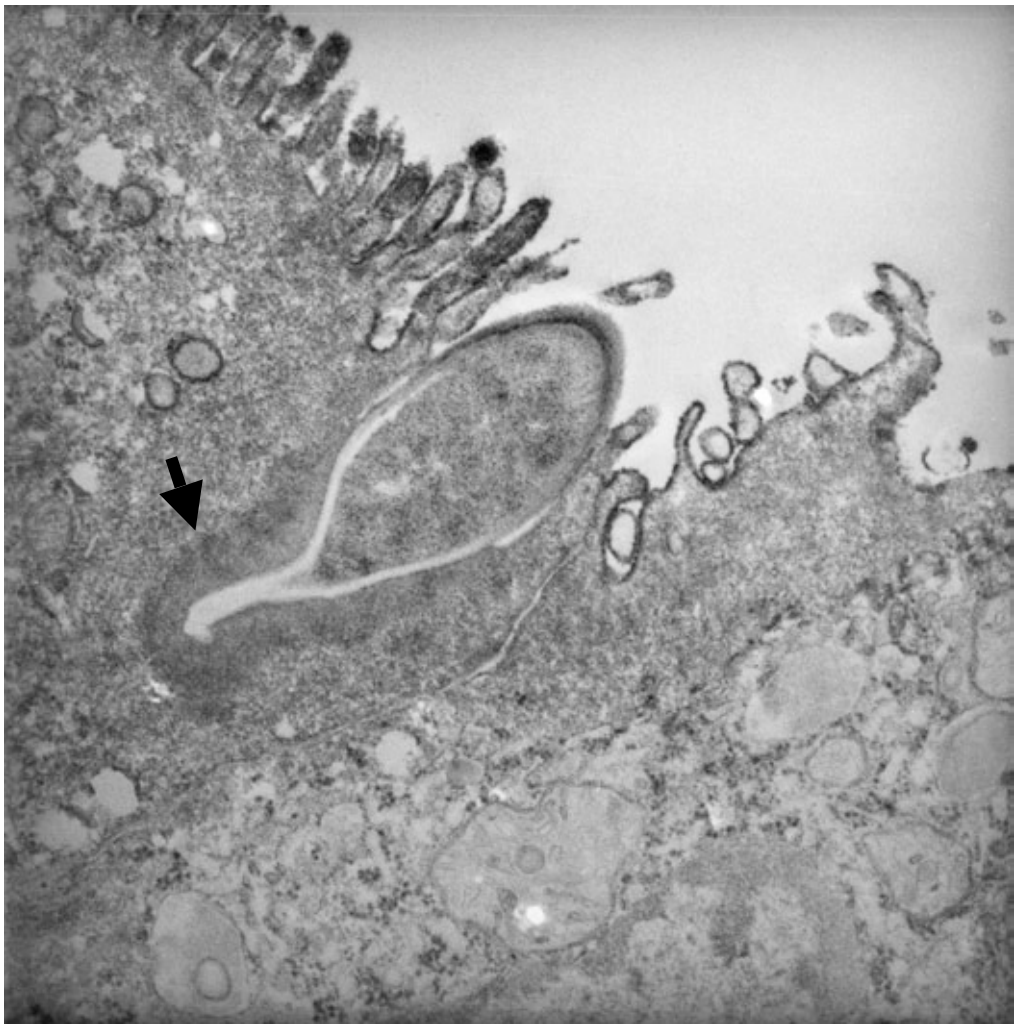

Figure 2. Transmission electron microscopic image of an epithelial cell containing an SFB holdfast. The brush border membrane of the epithelial cell is unaffected. The host cell has responded by accumulation of actin at the site of attachment (arrow).

ATTACHMENT AND EPITHELIAL RESPONSES

SFB adhere to the epithelial cells of the ileum with a special structure described as holdfast (Figure 2). The shape of this holdfast can vary from bean-, teardrop, to bulb-shaped (*Blumershine* and *Savage*, 1978). Using transmission electron microscopy, it is demonstrated that attachment of SFB causes an invagination of the plasma membrane and displacement of the microvilli at the site of attachment. The host cell responds to adhesion with the accumulation of polymerized actin, similar as seen after attachment of enteropathogenic *Escherichia coli*. In contrast to infection with pathogenic bacteria, the microvilli of the brush border remain intact (*Jepson* et al., 1993).

Attachment is not restricted to regular epithelial cells of the ileum:

SFB also adhere to the follicle associated epithelium of Peyer's patches in the ileum, specialized lymphoid organs as part of the mucosal immune system (*Jepson* et al., 1993; *Snel* et al., 1998). Within the follicle-associated epithelium, membranous cells (M-cells) are involved in the continuous sampling of antigens from the lumen. Although rarely seen, SFB are capable to adhere to M-cells of mice (*Jepson* et al., 1993; *Meyerholz* et al., 2002), and even extend from an M-cell into intimate association with an intra-epithelial mononuclear cell (*Meyerholz* et al., 2002).

Epithelial gene expression was examined by microarrays and compared to gene expression induced by the probiotic strains *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult after 3 days of mono-association of germfree mice (*Shima* et al., 2008). Most pronounced effects were found in the ileum where SFB far more than the two probiotics differentially expressed 942 genes (478 more than 2-fold upregulated and 464 more than 2-fold downregulated compared to germ-free animals) versus 362 for *Lactobacillus* (183 up and 179 down) and 264 for *Bifidobacterium* (75 up and 189 down). Surprisingly, the overlap in differentially expressed genes by these 3 strains was limited. It was found that the many of the upregulated genes belonged to the functional categories cell communication, defence and immunity, metabolism, and transport.

In ex-germ-free mice associated with SFB, fucosylation of asialo GM1 glycolipid occur in the small intestinal epithelial cells (*Umesaki* et al., 1995). Using the genomics approach, it was demonstrated that in particular, alpha(1-2) fucosyltransferase was induced in the gut epithelium after mono-association with SFB, but not after mono-association with a *Lactobacillus* or *Bifidobacterium* strain (*Shima* et al., 2008). Another interesting gene induced by SFB is pancreatitis-associated protein (PAP or RegIIIγ). This gene encodes a C-type lectin, and is even found to be induced after mono-association of germ-free severe combined immunodeficient (SCID) mice with SFB as an innate response to microbial colonization (*Keilbaugh* et al., 2005). A recent study described antimicrobial activity of the gene product, and suggested a role in gut homeostasis in order to maintain symbiotic host-microbe interactions (*Cash* et al., 2006).

Host epithelial cells in germ-free mice are known for their low expression of major histocompatibility complex II (MHC-II) molecules on the apical surface which is rapidly induced after conventionalization with a complete microbiota. After attachment, SFB can be phagocytised into the epithelial cells of the ileum and intracellularly processed by heterophagy (*Yamauchi* and *Snel*, 2000). Mono-association with SFB results in expression of MHC-II, but this is not seen after mono-association with related spore-forming bacteria from the genus Clostridium (*Umesaki* et al., 1995, 1999).

ROLE OF SFB IN HOST RESISTANCE

Several studies have suggested that SFB may increase the resistance of the host to infectious diseases. Newborns acquire adaptive and innate immunity through maternal sources, either via the transplacental route before birth or via the milk after birth. This process, referred to as passive immunity, provides a number of defence factors such as immunoglobulins, lactoferrin, lyso-

zyme, cytokines, and chemokines. In mice it is shown that the immune status of the dam also influence the development of the systemic and mucosal adaptive immune system of newborn animal (*Kramer* and *Cebra*, 1995). At this stage, the role of SFB is limited since they are not part of the intestinal microbiota of suckling mice.

During the weaning phase, high levels of SFB can be observed. Colonization of mice starts at about 3 weeks of age of the animals, at which the animals shift their diets from milk to solid food (*Blumershine* and *Savage*, 1978; *Koopman* et al., 1987). The high colonization level of SFB in weaning animals may competitively lead to reduced colonization levels of food-borne pathogens. Indeed, such an effect was observed in rats orally infected with *Salmonella enteritidis* (*Garland* et al., 1982). Here, a reduction of surface colonization by these pathogens was found in the presence of SFB. However, in another study, using either *S. enteritidis* or *Enterobacter cloacae* as the challenging microorganisms, the presence of SFB did not lead to significantly reduced translocation of pathogens (*Klaasen* et al., 1992b)

It is known that SFB are only abundantly colonizing the epithelium shortly after weaning in mice (*Davis* and *Savage*, 1974; *Klaasen* et al., 1992a; *Snel* et al., 1998) and for about 10 days after hatching in chicks (*Yamauchi* et al., 1990). During this period, the mucosal immune system is strongly stimulated, resulting in the induction of high levels of IgA plasma cells in the gut lamina propria and secretory IgA in gut secretions (*Klaasen* et al., 1993a), and αβ-T-cell-receptor bearing intra-epithelial lymphocytes (*Umesaki* et al., 1995). Since SFB colonization is temporary abundant in immunocompetent mice, whereas they persist in athymic nude mice (*Snel* et al., 1998) as well as in IgA-deficient mice (*Suzuki* et al., 2004), it is strongly suggested that it is the induced immune response leads to self-limiting colonization levels. A self-limiting response has been reported for translocation of the Gram-negative bacterium *Morganella morganii*, the number of translocating bacteria begins to drop with the onset of a specific IgA response while colonization of the intestinal lumen is unaffected (*Shroff* et al., 1995). The induction off IgA and other components of the mucosal immune system contribute to enhanced resistance to salmonella in mice. Young adult mice infected with *S. typhimurium* had a prolonged survival when mono-colonized with SFB compared to germ-free mice. Prolonged survival was not seen in mice mono-associated with *Clostridium innocuum* (unpublished data).

The influence of SFB on resistance to enteropathogens because of its high bacterial density during weaning and stimulation of the immune response post-weaning is schematically represented in Figure 3.

FACTORS INFLUENCING SFB COLONIZATION

Because of the role of SFB in maturation of the host immune system during the weaning period, it is of importance to understand factors either stimulating or suppressing the presence of these bacteria in the gut.

A few studies focussed on antimicrobial drugs in relation to SFB colonization. These studies are hampered by the inability to culture these bacteria *in vitro*. Penicillin was the first drug for which sensitivity of SFB was demon

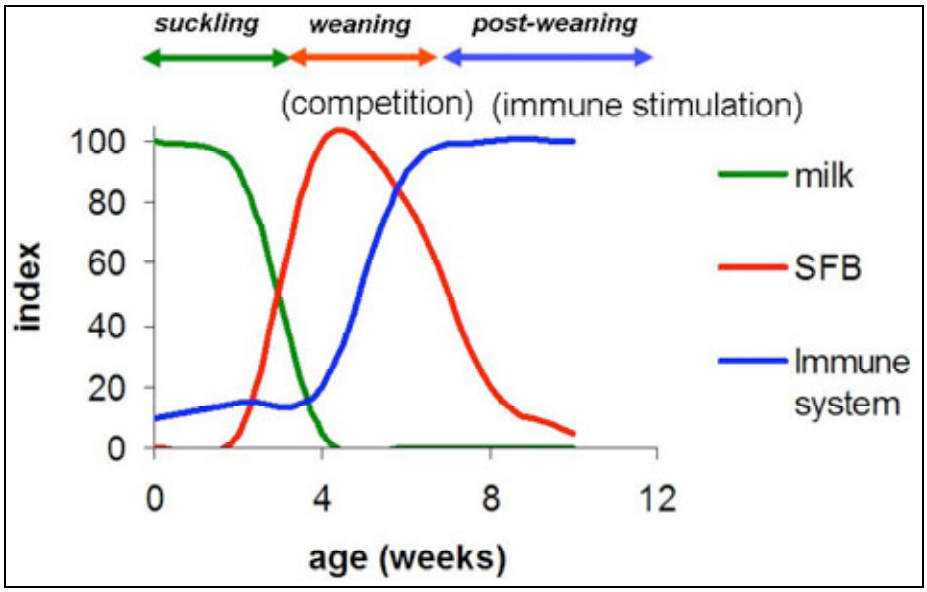

Figure 3. Schematic overview of the defence against enteropathogenic microorganisms. SFB contributes to competitive exclusion by high colonization levels during weaning and stimulates immune maturation after weaning.

strated (*Davis* and *Savage*, 1976). Only one study attempted to investigate antibiotic sensitivity of SFB in a systematic way and demonstrated that these bacteria were sensitive to amoxicillin, doxycyclin, ciprofloxacin, clindamycin, streptomycin, and cefotaxim when added to the drinking water (*Klaasen* et al., 1991b). Colonization was studied by microscopic evaluation of ileal scrapings of conventional animals, whereas at present mono-associated mice would have been available for these studies (*Klaasen* et al., 1991a). It has not been demonstrated whether antibiotic treatment of mono-associated animals would prevent maturation of the immune system. In conventional animals, treatment of mice with selective antibiotics is known to inhibit Th17 cell differentiation in the lamina propria and is accompanied by increase in Foxp3+ regulatory T cells (*Ivanov* et al., 2008).

Information on dietary influences of SFB colonization is scarce. In mice, the composition of the diet is a factor creating significant differences in SFB colonization. Nevertheless, an attempt to identify key dietary components by using purified diets failed since SFB colonization was completely inhibited (*Klaasen* et al., 1992c). So far, effects of macronutrients and their influence on SFB colonization are not systematically investigated.

Addition of red kidney beans (*Phaseolus vulgaris*) to the diet of 6-7 wk old mice stimulated colonization of SFB (*Klaasen* et al., 1991c, 1992c). Although phytohaemagglutinin (PHA) from red kidney beans is known to stimulate overgrowth of *Escherichia coli* (*Pusztai* et al., 1993), it is not likely that this lectin is responsible for the effect since also boiled red kidney beans, in which PHA was inactivated, led to the observed stimulation (*Klaasen* et al., 1991c). Besides, no increase of SFB was reported in a study in which purified PHA was added to the diet (*Banwell* et al., 1985).

CONCLUSIONS

Segmented filamentous bacteria are a group of bacteria that are found in the ileum of several mammals, birds and fish. Here they have a profound effect on maturation of the immune system. This does not only affect colonization of SFB itself, but also that of pathogenic microorganisms such as *Salmonella* and *E. coli*.

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THE DESIGN AND CONDUCT OF POPULATION STUDIES OF DIET AND HEALTH OUTCOMES

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SUMMARY

The association between diet and health and disease outcomes is best explored in large population-based observational studies. While a number of designs are available to study this relation, the prospective cohort design is least affected by biases, as it is not subject to recall or selection biases that affect case-control studies, nor is it plagued by the compliance problems of a randomized clinical trial. The most challenging problem of studying the association between diet and disease is the assessment of diet itself. Diet assessment is affected by measurement error, and there is no perfect way to assess diet. All diet assessment instruments that employ self-reports have considerable measurement error. Few biomarkers are known that reflect dietary intake well. While the search for additional biomarkers is ongoing, other methods of diet assessment using the Internet, telephone, and/or cameras are explored.

INTRODUCTION

The role of diet in health and disease has long been of interest and has been addressed in numerous studies. While much has been learned about the optimal diet from observational research during the past decades, the perfect study design to determine the role of diet in maintaining health is still debated. Since the assessment of diet remains challenging, numerous methods have been explored and new methods are under development.

REVIEW AND DISCUSSION

Several epidemiologic study designs can be employed to study the association between diet and disease.

Cross-sectional study

In a cross-sectional study, diet and disease are assessed at the same time. For example, in the National Health and Nutrition Examination Survey (NHANES) conducted in the United States, population-based samples of approximately 30,000 individuals are selected every few years and queried about their diet and other lifestyle factors (*Kant* et al., 1991). At the same time, the participants' health status is

assessed. In this cross-sectional approach, however, a time sequence cannot be established, i.e., it remains unknown whether an illness may be a consequence of the diet that is reported during the survey or whether the diet report may have been influenced by the disease.

Case-control study

In a case-control study, cases with the disease of interest and controls free of the disease of interest are sampled from the same source population. Cases and controls are asked to report their dietary intake typical for the time period preceding the disease in the cases. This study design is affected by two potential sources of error: Selection bias and recall bias. Since the cases report their past diet when they already have the disease, their disease status may affect the reporting of their diet. Differential reporting of diet by cases and controls introduces recall bias, which is a differential misclassification. Furthermore, if the controls are not appropriately selected, their diet may not be representative of the dietary intake of the source population that gave rise to the cases; this may introduce selection bias and distort the observed measure of association. Confounding by other dietary or lifestyle factors is an additional concern in an observational study.

Prospective Cohort Study

A prospective cohort study is not affected by the aforementioned biases. In a prospective cohort study, the diet of healthy individuals is assessed at the onset of the study and participants are followed over a substantial period of time until a certain number of them get the disease of interest. While a prospective cohort study is not affected by selection or recall bias, a problem arises if participants are lost to follow-up, i.e., contact with participants is not maintained during follow-up and therefore their health status is unknown. If a high participation rate over several years is accomplished, the cohort design is likely the best design available to study the association between diet and disease outcomes. An example of a prospective cohort study is the Nurses' Health Study, in which diet is assessed every four years and nearly 90% participation has been maintained for more than 30 years. Confounding remains a challenge also in cohort studies.

Randomized Clinical Trials (RCT)

While the role of nutrition in maintaining health could also be studied in a randomized clinical trial, which is not plagued by confounding, assigning a certain diet to individuals over a longer period of time is usually problematic. Even if individuals agree to adhere to a particular diet, maintaining such a diet over years, which would be necessary to study its effect on chronic disease outcomes, is virtually impossible. The recent example of the Women's Health Initiative demonstrates the difficulties of maintaining an assigned diet over several years (*Howard* et al., 2006). The successful randomization of diet is possible only if meals are provided to participants, which is practical only in the short term and with a limited number of participants. An example of a successful randomization of diet is the Dash Trial, which tested the effect of several diets on blood pressure (*Sacks* et al., 2001). Participants came to the hospital every day to consume one meal there and took the two remaining meals and between-meal snacks home. The Dash Trial was successful because all meals were provided and because a change in blood pressure with dietary intervention can be observed within a few weeks.

Assessment of Diet

The assessment of dietary intake in a human population is difficult, as diet is composed of many foods and drinks and most individuals do not remember what they consumed on any given day. Hence, it is only possible to obtain either a snapshot of an individual's diet at one time or to assess dietary preferences over longer time periods. When validating diet assessment instruments, they should be compared to a gold standard not affected by the same (correlated) measurement error, preferably a biomarker.

The 24-hour Recall

The 24-hour recall assesses diet during the previous 24 hours. While such a snapshot might capture a person's diet during one day, the previous 24 hours may or may not be typical in reflecting this individual's typical diet. A 24-hour recall is affected by measurement error if the individual does not remember all the items he/she consumed during the previous 24 hours and does not capture seasonal variation of diet. Obtaining several 24-hour recalls throughout a year might reduce this error component. Nevertheless, obtaining 24-hour recalls in a large population such as the NHANES study produces population means in the intake of dietary items that are approximately accurate (*Willett*, 1998).

7-day Diet Record

The 7-day diet record requires participants to maintain a diary of everything they consume and drink during a 7-day period. This approach has the advantage over the 24-hour recall that it captures diet during an entire week, including weekdays and weekends in which a person's diet usually varies. Moreover, maintaining a dietary record prospectively avoids the recall of diet and if carefully maintained can provide the best image of an individual's diet during one week. However, the 7-day diet record may not capture a person's typical diet if the diary week is not representative, and a one-week diary does not capture seasonal variation. Again, such measurement error may be reduced if the 7-day diet record is kept on four occasions throughout a year. Furthermore, some people may change their diet during the week of recording because they have to document everything they eat and drink. Hence, the 7-day diet record may not always accurately reflect a person's diet. In addition, computerization of the diet diaries for analysis in large observational studies is prohibitive (*Willett*, 2001).

Food Frequency Questionnaire

The food frequency questionnaire (FFQ) is the most widely used dietary assessment instrument in observational research. It consists of a pre-structured questionnaire that includes approximately 160 food items and up to nine response options for frequency of intake. The semiquantitative FFQ also provides portion sizes for most food items such as 1 glass, 1 cup, or 1 slice. Study participants are asked to report their average intake of a food or beverage per day, per week, or per month during the past year, or sometimes during a different time period, e.g., 6 months or 1 month. While the FFQ does not attempt to measure consumption of foods or beverages with high precision and is affected by measurement error, it captures dietary preferences reasonably well and separates high from low intake (*Willett*, 1998).

Alternative Diet Assessment Instruments

Attempts to improve on existing dietary assessment methods incorporate the use of existing technology. Recently, an Internet-based 24-hour die-

tary recall has been developed, which provides respondents with 8,000 foods to choose from enhanced by graphical display (National Cancer Institute). Information on time of consumption and preparation methods is also collected. Camera-assisted methods in which participants photograph all meals are not new (*Elwood* and *Bird*, 1983), but photos are now taken digitally and submitted to dieticians via mobile phone cards (*Wang* et al., 2006). Telephone-based methods are also being developed.

Biomarkers of Dietary Intake

Biomarkers that reflect dietary intake with high accuracy and precision are superior to self-reports that are prone to measurement error. Unfortunately, few such biomarkers for diet have been identified. Recovery biomarkers provide an estimate of absolute intake levels based on the metabolic balance between intake and excretion over a fixed period of time, and thus excretion levels are highly correlated with intake (*Bingham*, 2002). Examples of recovery biomarkers are doubly labelled water to measure energy expenditure and thus total caloric intake, urinary total nitrogen to estimate total daily protein consumption, and urinary total potassium to estimate total daily potassium intake (*Jenab* et al., 2009). Predictive biomarkers are also sensitive and time dependent, and show a dose-response relation with dietary intake, but their overall recovery is lower. The only known predictive biomarkers are 24-hour urinary sucrose and fructose levels, which are closely correlated with intake of sugars (*Tasevska* et al, 2005). Concentration biomarkers correlate with intakes of foods or nutrients, but the correlation is lower than that for recovery biomarkers and do not translate into absolute levels of intake (*Bingham* et al., 2008). Examples for concentration biomarkers are fatty acids, carotenoids, and other vitamins.

CONCLUSIONS

Prospective cohort studies are the preferred study design to assess the association between diet and health and disease in free-living populations. While the search for additional biomarkers of diet is ongoing and alternative dietary assessment methods are explored, most observational research currently relies on the use of the FFQ.

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THE ROLE OF THE INTESTINAL MICROBIOTA IN THE AETIOLOGY OF ALLERGIC DISEASES

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SUMMARY

The prevalence of allergic diseases, such as eczema, food allergy, hay fever and allergic asthma has been increasing in the past decades, predominantly in the Western world and particularly amongst children. An altered normal intestinal colonization pattern in infancy, which fails to induce immunological tolerance, could be partly responsible for this increase. The majority of epidemiological studies have indeed shown that the microbiota of allergic children differs from that of healthy children. Furthermore, the few prospective studies indicated that differences in the intestinal microbiota actually preceded the manifestation of allergic diseases, which strengthens the evidence for a causal relationship. Yet, results on which microbes might be involved in the aetiology of allergic diseases are inconsistent between studies and therefore no specific harmful or protective microbes can at present be identified. Furthermore, some studies indicate that low diversity and/or strain turnover rather than specific microbes may contribute to the development of allergic diseases.

The development of allergic diseases depends not only on environmental factors, like microbial stimulation, but also on genetic factors and it is likely to be an interaction of these which determines the allergic status of an individual. It is therefore most likely that the effects of certain microbes on allergy development differ according to the genetic susceptibility of an individual. To examine the influence of such host-microbial interactions there is a need for studies consisting of large populations in which both faecal samples for microbial analyses as well as blood or buccal swabs will be collected for genotyping. More knowledge on host-microbial interactions in allergic diseases could lead us to new concepts, such as the intervention with specific treatments to modulate the gut microbiota of infants at risk.

THE MICROBIOTA HYPOTHESIS IN ALLERGIC DISEASES

Atopic (or allergic) diseases, such as eczema, food allergies, hay fever and allergic asthma, are chronic inflammatory disorders caused by aberrant immune responses against common "innocuous" environmental antigens (allergens) in susceptible individuals (*Romagnani*, 2006). An enhanced T helper (Th)2 immune response and the elaboration of cytokines such as inter-

leukin (IL)-4, IL-13 and IL-5 contribute to the induction of these diseases (*Ngoc* et al., 2005).

The prevalence of allergic diseases has been increasing worldwide in the past decades, predominantly in the Western world and particularly amongst children (*Nakagomi* et al., 1994). Because this increase occurred much faster than the genetic constitution of any population can possibly shift (*Nowak* et al., 2004), it is generally believed that environmental changes associated with "western" lifestyles are responsible for the allergic epidemic.

Twenty years ago, David Strachan introduced the "hygiene hypothesis", which states that reduced exposure to infections during childhood result in aberrant immune responses to innocuous antigens later in life (*Strachan,* 1989, 2000). This hypothesis was based upon Strachan's observations that infants with higher number of siblings were at decreased risk for developing allergy. Although sibship size (*Karmaus* and *Botezan*, 2002; *Strachan*, 2000), and other indirect markers of microbial exposure such as rural and farm living (especially contact with livestock) (*von Mutius*, 2002) were consistently shown to be associated with a decreased risk of developing allergic diseases, studies on the association between bacterial and viral infections and allergy were less consistent (*Björkstén*, 2004; *Flohr* et al., 2005).

In 1998 Agnes Wold introduced an alternative interpretation of this hypothesis by suggesting that rather than a decrease in viral or bacterial infections, an altered normal intestinal colonization pattern in infancy, which fails to induce immunological tolerance, could be responsible for the increase in allergies (*Wold*, 1998).

The gut microbiota is indeed a key source of microbial driven immune regulation and tolerance induction in early life. Animals bred in a germ-free environment show low densities of lymphoid cells in the gut mucosa, and the specialized follicle structures are small, additionally circulating immunoglobulin levels are low. Immediately after exposure to microbes, the number of mucosal lymphocytes expands, germinal centres are formed and immunoglobulin-producing cells appear rapidly in follicles and in the lamina propria and there is a significant increase in serum immunoglobulin levels (*Butler* et al., 2000; *Falk* et al., 1998). Furthermore animal studies have shown that it is difficult to achieve oral tolerance in germ-free animals (*Sudo* et al., 1997) and that administration of lipopolysaccharides (a constituent of the outer membrane of Gram-negative bacteria) together with food antigens increases the tolerizing effect of feeding (*Kim* and *Ohsawa*, 1995). It seems therefore plausible that the gut microbiota composition (due to e.g. increased antibiotic use, changed diet) is involved in the pathogenesis of allergic diseases.

IMMUNOLOGICAL FRAMEWORK

The initial immunological explanation for the hygiene hypothesis was a lack of microbial antigen-induced immune deviation from the Th2 cytokine profile to a Th1 type profile, resulting in the development of enhanced Th2 cell responses to allergens (*Baker*, 2006; *Matricardi* and *Bonini*, 2000; *Romagnani*, 2004). However, this explanation did not take into account that the

prevalence of Th1-associated diseases, such as Crohn's disease, type 1 diabetes and multiple sclerosis, were also increasing and that chronic parasitic worm (helminth) infections which induce strong Th-2 responses and high IgE levels are not associated with an increased risk of allergy (*Yazdanbakhsh* et al., 2002).

An alternative interpretation conceives anti-inflammatory immune responses to be of fundamental importance in the development of mucosal and systemic tolerance (*Rautava* et al., 2005). These immunosuppressive mechanisms are orchestrated by regulatory T cell classes (Treg cells) that control (largely via the production of IL-10 and/or TGF-β) both Th1 and Th2 responses and hence the development of both atopic and autoimmune diseases (*Rautava* et al., 2005; *Rook* and *Brunet*, 2005a). Indeed the importance of a delicate balance between allergen-specific Treg cells and allergen specific Th2 cells in healthy and allergic immune responses to common environmental allergens was demonstrated in a study conducted by Akdis and colleagues (*Akdis* et al., 2004). Furthermore, a study on duodenal biopsies of healthy infants and infants with multiple food allergy, showed that the dominant mucosal abnormality was not Th2 deviation but impaired generation of TGF-ß producing Treg cells (*Perez-Machado* et al., 2003).

Relatively harmless organisms, including bifidobacteria, lactobacilli, but also helminths and saprophytic mycobacteria, may skew immune responses towards immunoregulation by inducing Treg cells, rather than eliciting a proinflammatory immune response.

For example, *Lactobacillus paracasei* has been reported to inhibit the secretion of both Th1 and Th2 cytokines, while inducing the development of a population of CD4(+) T cells producing TGF-ß and IL-10, reminiscent of previously described subsets of regulatory cells implicated in oral tolerance and gut homeostasis (*von der Weid* et al., 2001).

Lactobacillus reuteri and *Lactobacillus casei* have been shown to prime monocyte-derived DCs to drive the development of IL-10 producing Treg cells, through binding the C-type lectin DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (*Smits* et al., 2005). The Bifidobacterium genomic DNA has been reported to induce the secretion of IL-10 by PBMCs from healthy donors *in vitro* (*Lammers* et al., 2003).

The "microbiota hypothesis" proposes that the loss of exposure to these harmless microorganisms in the westernized environment might explain the increase in immunedysregulatory disorders (*Guarner*, 2006; *Rook* and *Brunet*, 2005b). The epidemiological findings and the experimental evidence available so far suggest that both the reduced immune suppression by Treg cells and the lack of immune deviation from a Th2 to Th1 profile are involved (*Romagnani*, 2004). Furthermore, the impact of the gut microbiota on the development of IgA antibody responses, which contribute in pathogen and allergen exclusion in the gut lumen, may also be involved (*Rautava* et al., 2004).

GUT MICROBIOTA AND ATOPIC DISEASES

Bengt Björkstén and his research group instigated the epidemiological research on the role of the intestinal microbiota in the aetiology of allergic diseases. Björkstén's group showed that the intestinal microbiota of healthy children

living in a country with a low prevalence of allergy (Estonia) differed from the microbiota of healthy children living in a country with a high prevalence of allergic diseases (Sweden). Lactobacilli and eubacteria were more prevalent in Estonian children, while *C. difficile* was more prevalent in Swedes (*Sepp* et al., 1997). Subsequently, this research group conducted a case-control study in which they showed that lactobacilli were less prevalent in allergic children compared to healthy children both in Sweden and Estonia (*Björkstén* et al., 1999). The disadvantage of case-control studies is their cross-sectional design, which makes it impossible to find out if differences in the intestinal microbiota actually preceded the development of allergic diseases. Therefore, Björkstén and coworkers additionally performed a birth cohort study including 44 newborns with a high risk of developing allergy (positive family history of allergy). Faecal samples were collected at the children's age 1 week and 1,3,6 and 12 months and analyzed using traditional bacteriological culture techniques and biochemical identification (*Björkstén* et al., 2001). The prevalence of bifidobacteria was consistently lower throughout the first year of life in children who developed allergic symptoms and sensitization, indicating that differences in intestinal microbiota actually precede the development of allergic manifestations. In the subsequent 5 years several, mostly cases-control, studies were published. Almost all these studies reported differences in the intestinal microbiota between allergic and non-allergic subjects. However, as mentioned before, case-control studies are limited by their cross-sectional design. In 2007 results from 2 large birth cohort studies, our own KOALA study and the ALLERGYFLORA project, were published.

The KOALA Birth Cohort Study

The KOALA Birth Cohort Study was the first large-scale prospective study in which the role of the gut microbiota in the aetiology of allergic disorders has been investigated.

Within the KOALA study 2834 pregnant women were recruited at 34 weeks of gestation. Beginning halfway during this recruitment of the cohort, faecal samples of infants (n=1176) at the age of 1 month were collected. During pregnancy and early childhood data on perinatal determinants of the child's health as well as on hygiene, infections, nutrition, child rearing, other life style characteristics and on allergic symptoms was collected for all members of the cohort by repeated questionnaires. A large number of children of whom faeces had been collected were also visited at home by a trained nurse at the age of 1 and 2 years for the collection of a blood sample and a clinical diagnosis of atopic dermatitis (*Kummeling* et al., 2005).

This study enabled us, not only to examine the association between the gut microbiota and allergic diseases, but also to examine the external factors influencing the composition of the intestinal microbiota.

Using quantitative real-time PCR, we found that infant feeding had a major effect on the gut microbiota of 1-month-old infants. Breastfed infants were less often colonized with bacteria other than bifidobacteria compared to formula-fed infants. Mode and place of delivery also appeared to be of great importance. Children born by C-section were less often colonized by *Bacteroides* spp., whereas they were significantly more often colonized by *Clostridium difficile*. Also hospitalization after birth resulted in an increased risk of becoming colonized by *C. difficile*, demonstrating the hospital environment as an important source of this bacte-

rium. As expected, children who received oral antibiotics in their first month of life had a strong reduction in obligate anaerobes (*Penders* et al., 2006a).

We also found strong support for the role of the gut microbiota composition in the development of atopic manifestations. The prevalence and counts of faecal *Escherichia coli* at the age of 1 month was significantly higher in infants who subsequently developed eczema within the first two years of life. Colonization with *Clostridium difficile* was positively associated with the subsequent development of eczema, recurrent wheeze and allergic sensitization at age 2 years (*Penders* et al., 2006b; *Penders* et al., 2007). Interestingly, two studies examining *Clostridium difficile*-specific immunoglobulin G also identified this bacterium as a risk factor for atopic diseases (*Linneberg* et al., 2003; *Woodcock* et al., 2002). Furthermore, a study on faecal short chain fatty acid-profiles showed that allergic children had higher levels of the rarely detected i-caproic acid, which has been associated with the presence of *Clostridium difficile* (*Bottcher* et al., 2000).

ALLERGYFLORA

Another large-scale prospective birth cohort examining the hypothesis that allergic sensitization and atopic eczema/dermatitis are influenced by the infant gut microbiota is the ALLERGYFLORA-project (*Adlerberth* et al., 2007). In Göteborg, London and Rome 324 children were recruited perinatally. A rectal sample was collected at age 3 days, whereas faecal samples were collected at 7, 14, 28 days and 2, 6 and 12 months and analyzed by traditional culture techniques. At the age of 18 months, the presence of atopic dermatitis was assessed as well as serum specific IgE to common food-allergens. This study reported a comparable "negative" impact of birth by C-section with delayed *E. coli* and Bacteroides colonization and increased colonization by Clostridium species as was found in our KOALA-study.

However, neither atopic eczema nor food-specific IgE by age 18 months were associated with time of acquisition of any particular bacterial group. In a second publication from this project, the microbial diversity of faecal samples collected at the age of 1 week was assessed by terminal restriction fragment length polymorphism (T-RFLP) and temperature gradient gel electrophoreses (TTGE) (*Wang* et al., 2008). It was demonstrated that the microbial diversity in early faecal microbiota was reduced in infants who subsequently developed atopic dermatitis.

An overview of the studies conducted in the past decade on the association between the intestinal microbiota and allergy is given in Table 1. Although most of the studies, conducted so far, showed an association between the intestinal microbiota composition and allergic symptoms and/or sensitization, results are far from being consistent and no specific harmful or protective microbes can be identified yet.

It is unlikely that publication bias and false positive findings due to multiple comparisons in several studies can completely explain the high percentage of studies that report an association between the intestinal microbiota and allergies.

So why do the majority of studies report an association, but are results far from consistent? It is likely that this relates to the large differences in methodological aspects between studies. Current studies are very difficult to compare due to differences in study design, differences in the bacteria under study and the techniques used to identify them and probably most important differences in the age at which

Table 1: Overview of studies on the association between the intestinal microbiota composition and allergy

Study	Allergic outcome	Study population	Design	Main results for allergic compared to non-atopic subjects
Björkstén et al., 1999	AD & SPT+	27 cases & 35 controls (aged 2 yr.)	Case-control	Lower prevalence of lactobacilli
Björkstén et al., 2001	AD/SPT +	44 newborns (until age 2 yr.)	Birth cohort	Lower prevalence of bifidobacteria
Kirjavainen et al., 2001	AD	27 cases & 10 controls (aged 5-13 mo.)	Case-control	No differences in concentrations of specific genera
Ouwehand et al., 2001	AD & SPT+	7 cases & 6 controls (aged 2-7 mo.)	Case-control	Higher prevalence of *Bifidobacterium adolescentis* Lower prevalence of *Bifidobacterium bifidum*
Kalliomaki et al., 2001	SPT+	76 newborns (until age 1 yr.)	Birth cohort	Lower bifidobacteria : clostridia ratio (at age 3 weeks)
Watanabe et al., 2003	AD	30 cases & 68 controls (minors)	Case-control	Higher prevalence of *S. aureus* Lower counts of bifidobacteria
Matsumoto et al., 2004	Severe AD	11 cases & 14 controls (adults)	Case-control	Lower total counts and total anaerobes. Higher proportion of enterobacteriaceae Higer content of sIgA
Murray et al., 2005	Recurrent wheeze & SPT+	33 cases & 33 controls (aged 4 yr.)	Case-control	No differences between cases & controls in lactobacillus and bifidobacterial colonization
Sepp et al., 2005	AD, asthma or allergic rhinitis	19 cases & 19 controls (aged 5 yr.)	Case-control	Lower prevalence and proportion of bifidobacteria Higher proportion of clostridia
Mah et al., 2006	AD	21 cases & 28 controls (aged 3 yr.)	Case-control	Higher counts of LAB Lower counts of bifidobacteria and clostridia.
Penders et al., 2007	Eczema , spIgE or recurrent wheeze	957 newborns (until age 2 yr.)	Birth cohort	Higher prevalence & counts of *E. coli* in infants who subsequently developed eczema Higher prevalence of *C. difficile* in infants who subsequently developed eczema, recurrent wheeze and/or became sensitized
Penders et al., 2006b	Eczema & spIgE	26 cases & 52 controls (aged 1 yr.)	Nested case-control *	Higher prevalence of *E. coli* No differneces in total bacterial profiles No difference in bifidobacterial counts & species composition
Adlerberth et al., 2007	AD, sp IgE	324 newborns (until age 18 mo.)	Birth cohort	No differences in time of acquisition of any bacterial group

AD: Atopic dermatitis; SPT: Skin Prick Test; spIgE: specific serum IgE to one or more allergens; FISH: Fluorescence *in situ* hybridisation.
* (Nested) case-control studies with a prospective design (faecal samples collected prior to the onset of allergic symptoms and sensitization).

Table 1 (continued): Overview of studies on the association between the intestinal microbiota composition and allergy

Study	Allergic outcome	Study population	Design	Main results for allergic compared to non-atopic subjects
Songjinda et al., 2007	AD, asthma, food allergy	8 cases & 7 controls (aged 2 yr.)	Nested case-control*	Higher population of Bacteroidaceae
Stsepetova et al., 2007)	AD, asthma, allergic rhinitis	20 cases & 20 controls (aged 5 yr.)	Case-control	Lower bacterial diversity Higher prevalence of *Bifidobacterium adolescentis* Lower prevalence of *B.catenulatum/pseudocatenulatum*
Gore et al., 2008	Eczema	37 cases & 34 controls (aged 3-6 months)	Case-control	No difference in total bacterial profiles Higher prevalence of *Bifidobacterium pseudocatenulatum*
Wang et al., 2008	AD	15 cases & 20 controls	Nested case-control*	Lower bacterial diversity
Verhulst et al., 2008	wheeze	154 newborns (until age 12 mo.)	Birth cohort	Lower clostridial concentrations Higher total anaerobes
Sjögren et al., 2009)	Symptoms (AD, asthma or allergic rhinitis) & SPT+	47 newborns (until age 5 yr.)	Birth cohort	Lower prevalence of lactobacilli group I Lower prevalence of *C. difficile* Lower prevalence of *B. adolescentis*

AD: Atopic dermatitis; SPT: Skin Prick Test; spIgE: specific serum IgE to one or more allergens; FISH: Fluorescence *in situ* hybridisation.
* (Nested) case-control studies with a prospective design (faecal samples collected prior to the onset of allergic symptoms and sensitization).

the intestinal microbiota is being studied.

The timing of exposure to environmental factors is essential to promote beneficial or harmful effects regarding the development of allergic diseases. It has been suggested that the most important "window of opportunity" for immune education seems to be in early life, when the maturation of the immune system is not yet completed and is still building up immune tolerance against food and microbial antigens (*Björkstén*, 1999; *Strachan*, 1989, 2000). Based upon this critical window period, it thus seems unlikely that perturbations in the intestinal microbiota beyond infancy may still have an effect on the aetiology of allergic diseases. It is more likely that these differences reflect disturbances in the gut microbiota already present in early life or reflect perturbations caused by the allergic disease that had manifested already (reverse causation).

Birth cohort studies quantifying the intestinal microbiota in early life and relating this to allergic manifestations later on in childhood are therefore the most powerful studies.

Another question that has not been answered yet is the differential effect of different faecal Lactobacillus species in the development of allergic diseases. The quantification of the gut microbiota has mainly relied on the quantification of bacteria at the genus level, such as *Lactobacillus* spp. Most studies found no association between the presence and quantity of total lactobacilli and allergy. It is, however, well known that different species of lactobacilli induce distinct and even opposing immune-responses (*Christensen* et al., 2002). Thus it is of special importance to unravel the potential species-specific effects of lactobacilli in the aetiology of allergic disorders, since it will gain more insight into the candidate species proficient as probiotics in the treatment or prevention of these disorders.

New upcoming birth cohort studies using molecular techniques to study the intestinal microbiota and assessing a broad range of allergic outcomes and immune parameters will probably gain more insight into the role of the gut microbiota in the aetiology of allergic diseases. Furthermore, the use of more recently introduced techniques such as microarrays and pyrosequencing within such studies will result in a far more detailed examination of the intestinal microbiota composition.

CANDIDATE GENES

The development of allergic diseases, including asthma, depends, however, not only on environmental factors (like microbial stimulation), but also on genetic factors and it is likely to be an interaction of these, particularly in early life, which determines the allergic status of a person (*Koppelman*, 2006, *Postma* et al., 2005). It is most likely that the effects of certain microbes on the development of allergy and asthma therefore differ according to the genetic susceptibility of an individual. The separate research on genetic (genetic epidemiology) and environmental influences (environmental epidemiology), as in most studies so far, has considerably hindered the understanding of the role of these influences in determining complex diseases like asthma and allergy. To get a better understanding of the biological importance of genetic and environmental factors, upcoming studies should include the interaction between these factors in relation to the disease phenotype.

Microbes are recognized by the innate immune system using pattern recognition receptors (PRRs). Interestingly Single Nucleotide Polymorphisms (SNPs) in PRR genes have been associated with allergy and asthma (*Eder* et al., 2004; *Fageras Böttcher* et al., 2004; *Koppelman* et al., 2001; *Yang* et al., 2006). Polymorphisms in these PRR encoding genes can alter the immune responsiveness of the host to microbial agents and may indicate the development of aberrant immune responses that are associated with immune-mediated diseases such as allergic diseases. Examples of PRRs are *CD14*, the toll-like receptors (TLRs), the NOD-like receptors (NLRs) and several C-type lectins.

CD14 is, together with *TLR-4*, involved in the recognition and signal transduction of bacterial endotoxin, a major component of the bacterial cell wall of Gram-negative bacteria. *CD14* does not have a transmembrane receptor domain, but contributes to the affinity of the interaction between the microbial products and TLRs. *TLR4* more selectively forms the receptor for endotoxin. Downstream effects of *CD14*/*TLR4* receptor activation include the release of cytokines, such as IL-10 and IL12, and the activation of regulatory T cells (*Vercelli*, 2003a). The *CD14* gene has several SNPs, the most important one being *CD14*/-159 C-T SNP (also called *CD14*/-260) localized in the promoter region. This SNP affects the transcription rate of the *CD14* gene (*Koppelman*, 2006, *LeVan* et al., 2001). Genetic associations of this -159 C-T polymorphism with markers of allergy have been shown in several studies (*Koppelman* et al., 2001). However, in some studies, the *CD14*/-159 C allele was associated with allergic phenotypes, whereas in other studies the T allele was. Finally other populations reported no association between the *CD14* genotype and allergy (*Kedda* et al., 2005). Vercelli explained these apparent contradictory results by proposing the "endotoxin switch" (*Vercelli*, 2003b). Different levels of (i.e. high or low) endotoxin exposure would trigger different host responses, resulting in either Th1 or Th2 type responses. The *CD14* genotype may shift this endotoxin response curve, highlighting the importance of studying the combined effect of endotoxin exposure and *CD14* genotype.

Two SNPs, A896G (Asp299Gly) and C1196T (Thr399Ile), in the *TLR4* gene have been associated with LPS hyporesponsiveness in primary human epithelial cells and alveolar macrophages *in vitro*, and with airway hyporesponsiveness to inhaled endotoxin *in vivo* (*Yang* et al., 2006). In a study among Swedish school children, the Asp299Gly polymorphism was associated with a 4-fold higher prevalence of asthma (*Fageras Böttcher* et al., 2004).

Polymorphisms in the *TLR2* gene have also been associated with the frequency of allergies and asthma (*Eder* et al., 2004). *TLR2* recognizes bacterial lipopeptides and lipotheichoic acid, which are abundantly found in cell walls of Gram-positive bacteria. Studies using murine models on the interaction between *TLR2* stimulation and allergy have provided contradictory data. Initial studies using murine models of allergic asthma reported that *TLR2* ligands administered during the sensitization period led to enhancement of Th2-mediated allergic inflammation. Other studies suggested that *TLR2* stimulation inhibits Th2-type responses and allergic airway inflammation. In a recent study stimulation of blood mononuclear cells of allergic individuals with *TLR2* ligands inhibited Th2 responses (*Taylor* et al., 2006).

The gene encoding the C-type lectin, dendritic cell-specific ICAM-3-

grabbing nonintegrin (*DC-SIGN*), is another candidate gene. Different species of lactobacilli have, for example, shown to induce distinct and even opposing dendritic cell (DC) responses with regard to their Th1/Th2/Treg-driving capacity. Recently it has been shown that certain, but not all, lactobacillus species induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function by targeting *DC-SIGN* (*Smits* et al., 2005). This could explain how certain lactobacillus species might exert beneficial effects in the treatment of allergic disorders.

Other candidate PRR encoding genes that have been suggested to be associated with asthma and allergy are the Mannose-binding lectin gene, *TLR6* and *TLR10* (*Yang* et al., 2006).

HOST-MICROBIAL INTERACTIONS

TLR and *CD14* gene association studies with asthma and allergy are suggestive but have not been fully replicated. One of the most likely explanations for differences between studies is difference in the level of microbial exposures interacting with the PRRs.

Support for the concept that the interactions between these innate immunity genes and microbes play a central role in the pathogenesis of asthma and allergies is derived from the few studies in which both (markers of) microbial exposures and SNPs in genes that encode for proteins that interact with these exposures were assessed. For example, in two independent populations, a SNP in toll-like (TLR) 4 (Asp299Gly) that disrupts *TLR-4*-mediated LPS signalling was associated with a lower prevalence of bronchial responsiveness and allergy, respectively, but only in subjects heavily exposed to endotoxin (*Eder* et al., 2004; *Werner* et al., 2003). Several studies have reported that the association between a functional SNP in the promoter region of *CD14* (CD14/-260C-T) and total serum IgE levels is modified by exposure to microbial products/endotoxin (*Simpson* et al., 2006).

Genetic variants within *NOD1*/ *CARD4* also appear to be important determinants of allergy susceptibility. Recently, the Allergy and Endotoxin (ALEX) study reported that SNPs in *NOD1*/ *CARD4*, an intracellular PRR that interacts with muropeptides found in common Gram-negative bacteria, modify the protective effect of farming (*Eder* et al., 2006).

So far, however, no gene-environment studies have been performed with respect to the interaction between candidate genes and the most important source of microbial stimulation, the gut microbiota.

More knowledge of host-microbial interactions in asthma and allergy could lead us to new concepts, such as the intervention with specific treatments to modulate the gut microbiota of infants at risk.

CONCLUSION

Most studies show an association between the intestinal microbiota composition in early infancy and allergic diseases. Moreover, the few prospective studies have shown that differences in the gut microbiota composition precede the development of allergic symptoms, which strengthens the evidence for a

causal relationship. However, no specific microbes or perturbations in the intestinal microbiota can be identified yet. Different microbes have been linked to allergies within the different studies. Furthermore, some studies indicate that a low diversity and/or strain turnover rather than specific microbes may contribute to the development of allergic diseases.

To get more insight into the role of the gut microbiota in the development of allergic diseases, more large-scale prospective cohort studies are necessary. Especially, birth cohorts in whom faecal samples will be collected at regular time-points during the first year of life will probably add to our current knowledge.

In addition, allergic diseases are complex diseases caused by the interplay of both genetic and environmental factors. The separate research on genetic (genetic epidemiology) and environmental influences (environmental epidemiology), as in most studies so far, has considerably hindered our understanding of the role of these influences in these diseases. So far, no studies have examined the interaction between host factors, such as genetic variations in PRR encoding genes, and the most abundant source of microbial stimulation, the intestinal microbiota.

To examine the influence of such host-microbial-interactions there is a need for studies consisting of large population in which both faecal samples as well as blood or buccal swabs will be collected for genotyping. More knowledge on host-microbial interactions in asthma and allergy could lead us to new concepts, such as the intervention with specific treatments to modulate the gut microbiota of infants at risk.

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INTESTINAL MICROBIOMICS: NOVEL INDICATORS OF HEALTH AND DISEASE

SUMMARY OF THE SEMINAR DISCUSSION

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For a long time, we may have thought that we had a good view on our intestinal microbiota. The bacterial community, which inhabits the human gastrointestinal tract, is characterized by its high density and diversity. The colon contents support an estimated 1000 different species, with numbers as high as 10^{10} or 10^{11} bacteria/gram of content. These bacteria are in a continuous interaction with each other, and with the host, comprising a highly complex ecosystem. The ecosystem as such contributes to host health as an aid in the digestion of foods, by production of vitamins, supporting the maturation of the immune system, aiding in the digestion of foods, and forming a barrier for colonization of pathogenic bacteria.

Our understanding on the microbial ecosystem has led to practical applications. Food products are on the market that claim to alter the microbial composition in the gut and thereby promoting human health. Prebiotics and probiotics are the best examples for this. Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health. The prebiotic concept was formed in the mid-nineties, when we had a rather premature view on the microbiota and its composition. At that time, bacteria were divided in harmful, neutral and beneficial species; the latter being promoted by prebiotic ingredients. Whereas prebiotics are substrates for beneficial bacteria, the probiotic concept is based on the consumption of beneficial bacteria themselves. Probiotic bacteria have an added value to the functionality of the normal microbiota. Although some pre- and probiotic products may be a commercial success, most health claims are under debate and so far, the medical world has not embraced the use of pre- and probiotics by implementation in standard protocols.

Despite years of research, and in contrast to the above, we seem to know relatively little on functionalities or species that really matter to health. In his opening presentation of the Old Herborn University Seminar, *Michael Zasloff* has stressed the importance of host-microbe interactions for some intestinal (mainly invertebrate) ecosystems, but also the apparent lack of hard evidence for a similar effect in humans. In many cases, only weak associations between microbiota composition and disease parameters could be made without a clear view on causality. To what extent individual species of the intestinal microbiota make a difference, or can be used as markers for a healthy microbiota is largely unknown.

The discussion session, of which the summary is presented here, started with an overview of the old concept of harmful and beneficial bacteria, and a description of the prebiotic concept. In addition, the following questions were raised:

- Do we have evidence that the microbiota composition reflects our health status?
- If the microbiota composition really matters, how can we turn this into practical tools for health care specialists?

Participants were challenged to provide examples on host- microbe interactions that are relevant for human health.
It is only recently that new techniques in molecular biology allow the detection of microbial species that are either difficult or as yet impossible to culture. Techniques such as fluorescent *in situ* hybridisation and denaturing gradient gel electrophoresis revealed first insight in intestinal microbiota composition and dynamics during health and disease. Novel techniques like microarray technology using chips spotted with probes that recognize different species of the human intestinal tract, and sequencing technology such as barcoded P454 pyrosequencing will provide further insight in the dynamics of microbiota composition.

During the discussion, it became clear that there are many factors that determine intestinal microbiota composition, some of which are of importance even directly after birth. Several of these factors were addressed during the various presentations of the seminar. Each author was asked to highlight the take home message of its presentation. These key messages, in the order of the seminar program were:

Lars Engstrand: Methodologies have become available today that allow us to assess the microbiota composition in very large cohort studies. Barcoded P454 pyrosequencing is a most promising technique that has been used to determine shifts in faecal microbiota, and is currently used in several other projects.

Joel Doré: Comparison of the microbiota of a group of volunteers reveals a number of species that are always present. This is referred to as the core microbiome. To what extend the core microbiome is conserved is subject of debate. Prevalence rather than absolute conservation is also a key-subject.

Michiel Kleerebezem: The small intestinal tract is the organ where most of the interactions between microbiota and host take place. We should therefore not forget this organ in our studies. In addition to the presence of species and their DNA, also the physiological state of these microbes is of importance.

Merete Eggesbø: Microbial colonization directly after birth likely has an impact on the health status of the host. In light with the hygiene hypothesis, Caesarean delivery is indirect a model for a distorted microbiota and has its impact on problems in later life.

Harro Timmerman: A Commensal Rat Ileum Bacterium (CRIB; spore former related to *Clostridium*) is found in a model for pancreatitis. Colonization levels were inversely associated with damage to the pancreas, which makes this a potential biomarker for a healthy microbiota.

Harry Flint: Core species are found in human subjects, the top ones actually described by *Moore* et al. (1996) after cultivation of these species. It suggests a functional redundancy, but this is probably not the case since ecological ability to maintain a high population level should come with a specific advantage. Cluster IV ruminococci are stimulated by starch in some individuals but not in all, as is often seen for dietary constraints. In non-responders, starch is detected in faeces.

Michael Blaut: Correlations between microbiota and health status can be seen but we need to be cautious in the point we make. There is a need to provide geneticists with better perspectives on key genes when relating microbiota functions and health, and we need reductionist models for this. A whole range of very good methods is available but need to be combined. This can fill the gaps between phylogeny and function in a few cases.

Hans Snel: There are organisms that have developed an intimate relation with host, as illustrated in mice with segmented filamentous bacteria. Adherence of these bacteria promotes priming of immune system as seen by T-cell proliferation and s-IgA production. This activation in turn reduced the number of adhering segmented filamentous bacteria.

John Penders: The host genotype is of importance when making associations between microbiota and health of the host. Early detection of (non-tox) *Clostridium difficile* is associated with all later outcomes of allergy, but this does not necessarily be a direct interaction between *C. difficile* and the host. An answer may come from prospective birth cohort studies, with possible later treatment as case-control sets.

Taken these key messages together, it becomes clear that the technology is getting in place to determine microbiota composition in great depth and at various parts of the gastrointestinal tract. These techniques have given us a first impression of important species (e.g. the concept of a human core microbiome, or presence of commensal rat ileal bacteria and segmented filamentous bacteria in rodents). We also get a better view on dynamics, and on factors like diet, antibiotic therapy and specific diseases that determine the final composition. Nevertheless, we still have difficulties in defining a healthy microbiota.

As a next step in the discussion, the concept of eubiosis (balanced microbiota) and dysbiosis (disturbed microbiota) was discussed. Eubiosis is based on both composition and dynamics of the microbiota. Regarding composition, we agreed that each adult faecal microbiota contains

- a few predominant conserved phyla (in varying relative proportions between subjects);
- over 1000 predominant species;
- a majority of species without cultured representatives;
- a majority of subject-specific species that are not necessarily being found in other subjects.

The core microbiome concept still needs further refinement. Regarding dynamics, this is highly dependent on the level of resolution of the analysis. However, the following characteristics are present:

- stability / resistance to change;
- resilience following stress (e.g. antibiotic therapy). To what extent the resilience is complete is still an open question.

There are so far no objective and generally accepted measures to describe dysbiosis. The time of sampling and other methodological aspects are important factors to consider. For Crohn's disease we seem to be closer to a general understanding of dysbiosis than for ulcerative colitis or allergies. Caesarean delivery is an interesting context promoting some dysbiosis.

Biomarkers for eubiosis are hardly present, and those available do require strengthening of evidence. Also biomarkers for microbiome functions rather than species are hardly present. As an example, one of the functions of the microbiota is to provide coloniza-

tion resistance, for which no adequate markers exist as yet. Some functions derive from single microbial species. There is still much to expect in this area, but we need more isolates for functions that have no representatives.

Early work on microbiota composition made a division between harmful and beneficial bacteria. This division is completely abandoned. Even pathogenic bacteria such as *Salmonella* spec. can be found in the intestines of apparently healthy persons. At present, there is no way to relate microbiota composition to a healthy state in absolute terms. There are, however, expectations to relate microbiota to disease risk. One example is the study of *Joel Doré* who presented data on the presence of *Faecalibacterium prausnitzii* that is associated with the absence of a relapse in Crohn's disease. At the moment, this is one of a few (if not the only) examples of microbiota elements that can serve as indicators for health or disease. This illustrates that it is possible to relate microbiota elements (present or missing) to disease risk. We do not know yet whether those elements are causally related to health or disease. No complete demonstration for that is yet available. Some bacterial "metabolites" are signalling to human cells and induce specific responses, and some "unknown signal metabolites" are protective against certain diseases in models.

What is needed?

To validate hypotheses on host-microbe interactions, the following studies and materials are necessary to draw proper conclusions:

- Prospective studies are needed since these allow a clear discrimination between microbial factors that precede a disease state, and factors that are the consequence of a disease state. Of critical importance for such studies are standardized sample collection and timing of collection. Some studies require collection of faecal material from children (e.g. for studies related to development of allergies) whereas other may preferably sample from adults (e.g. for studies related to colon cancer risk).
- There is a need for more studies on small intestinal microbiota since most host-microbe interactions take place in this organ. As this is the organ for immune sampling, bacterial interactions related to immunodevelopment likely are found here.
- Effect of bowel cleansing should be assessed as a way to study the impact of reduced bacterial numbers.
- There is a need for more studies on functionalities, e.g. butyrate production, rather than a focus on bacterial species. Most functionalities are not bound to one bacterial species, and therefore a change in microbiota composition at the species level does not necessarily result in changes in functionalities of the microbiota.
- We should biobank faecal and intestinal samples to have those available to test future hypotheses. So far, studies in which intestinal or faecal material is collected have not done so. The impact of storage conditions is largely unknown, and has to be considered since these samples need to be available for both microbial and biochemical analyses.

We know that some initiatives underway. In Europe together with China the MetaHIT project has been initiated, the United States has launched the Human Microbiome project, and Canada has the Canadian Microbiome Initiative. Also other national initiatives take place. We should learn from the out-

come of these projects to what extent the human microbiota contains elements that can be predictive for present and future health or disease.