Old Herborn University Seminar Monograph

30. THE MAGNIFICENT MICROBIOME - FUTURE ASPECTS

EDITORS:

PETER J. HEIDT
THOMAS C.G. BOSCH
TORE MIDTVEDT
VOLKER RUSCH

Old Herborn University Seminar Monograph 30

ISBN 3-923022-42-5
ISSN 1431-6579

Old Herborn University

Old Herborn University Foundation
Postfach 1765
D-35727 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 - 921101

Dirk van der Waaij
1931-2016

Dirk van der Waaij was the originator and one of the founders of the Old Herborn University Seminars. He had for a long time the wish to organise yearly meetings on the subject "microflora and immune system" and to publish the proceedings of these meetings in a series of monographs. This idea became reality in 1985 when Dirk and I attended the 10th International Symposium on Intestinal Microecology in Minneapolis, Minnesota (USA). During this symposium we had a meeting with Volker Rusch in the bar of our hotel and Dirk discussed his ideas on these yearly meetings with us. Volker had organised the Symposium on Intestinal Microecology the year before in Herborn, Germany as an honour to the University of Herborn (that was founded in 1584 by William I "the Silent", but closed by Napoleon in 1812). Volker recognised in Dirks idea possibilities to restore the academic environment in Herborn and during that evening the "Old Herborn University Seminars" were born and the ideas of Dirk became reality.

Dirk, who was born in Semarang (in the Dutch East Indies, nowadays Indonesia), did spend 3 years (from 1942 until 1945) from his youth in Japanese concentration camps. During the last year of the war he was "hospital-care-taker-assistant" in a "boys concentration camp" housing boys of ten years and older. This might be the reason that, after finishing high school in the Netherlands, he decided to study medicine in Leiden. After completing his medical studies he performed his PhD studies in the Laboratory for Parasitology of the Leiden

University and received his PhD title cum laude after defending his thesis entitled "Development and transmission capabilities of *Toxoplasma gondii* during experimental infection with chronic course in mice". His studies included the role of innate immunity as well as adaptive immunity and can be considered as the start of his lasting interest in the relation between the microflora and the immune system, which was and still is one of the main subjects of the Old Herborn University Seminars.

After finishing his PhD studies, Dirk worked from 1959 until 1975 as head of the Department of Microbiology in the Radiobiological Institute TNO in Rijswijk, the Netherlands, where he started and maintained germ-free and SPF rodent colonies and where he also had the possibility to work with other laboratory animal species, such as beagle dogs and rhesus macaques. Here, Dirk developed his ideas on how to manipulate the microflora in order to prevent infections in immunocompromised patients as well as graft-versus-host disease, a serious immunological complication after allogeneic bone marrow transplantation. His concepts of complete and selective decontamination of the gastrointestinal tract, reverse isolation by the use of laminar airflow systems, as well as the concept of "colonisation resistance" have had great influence on the treatment of patients with leukaemia and other disorders of the haematopoietic system. He was the initiator of the Gnotobiotic Project Group of the European Organisation for Research on the Treatment of Cancer (EORTC), which performed several international multicentre studies on the effect of selective decontamination of the gastrointestinal tract and nursing under reverse isolation conditions on the survival of leukaemia patients during intensive chemotherapy.

In 1975, Dirk was appointed Professor in Bacteriology and Serology at the Department of Medical Microbiology of the University of Groningen where he worked until his retirement in 1992. He had many PhD students, working again on the relation between the microflora and the immune system, as well as on methods to characterise the microflora. Many of those students were speakers during the different Old Herborn University Seminars, in this way also disseminating the original ideas of Dirk.

During the years that he was active in organising the Old Herborn University Seminars, he had every year new ideas on how the program could give insight in, what he called, the white spots: the parts of the puzzle that were missing to understand the important influence of the microflora on immune and other functions of the host. After his retirement in 1992, Dirk continued to be a member of the Old Herborn Seminar Committee until he decided in 2009 that it was time to make place for the younger generation.

In the years after 2009, Dirk developed health problems that made it difficult for him to travel and thus to attend the seminars, but he was present at the 25th seminar during which his important role in founding and continuing the Old Herborn University Seminars was highlighted.

We miss him greatly and will remember him as a driven scientist whose original ideas and research still have a great influence on basic science as well as on the medical field. We express our condolences to his wife Hillie and his family.

Peter J. Heidt

Contents

Contents (continued)

Contents (continued)

Contents (continued)

Participating Authors

Prof. Dr. Dr. h. c. Thomas C.G. Bosch, Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstraße 40, D-24098 Kiel, Germany.
(E-mail: tbosch@zoologie.uni-kiel.de).

Merete Eggesbø, M.D., Ph.D., Norwegian National Public Health Institute, P.O. Box 4404, Nydalen, N-0403 Oslo, Norway.
(E-mail: merete.eggesbo@fhi.no).

Andrew L. Kau, PhD, Center for Women's Infectious Disease Research, and Center for Genome Sciences and Systems Biology, Washington University School of Medicine, Campus Box 8122, 660 S. Euclid Ave., St. Louis, MO 63110, USA.
(E-mail: akau@wustl.edu).

Dennis Lang, PhD, Senior Project Manager, MAL-ED, Foundation for the National Institutes of Healt (NIH) and The Fogarty International Center, NIH, 9650 Rockville Pike, Bethesda, MD 20814, USA.
(E-mail: Lang4@FNIH.org).

Francois Leulier, PhD, Institut de Génomique Fonctionnelle de Lyon (IGFL), Ecole Normale Supérieure de Lyon, CNRS UMR 5242, Université Claude Bernard Lyon 1, 46 Allée d'Italie, 69364 Lyon Cedex 07, France.
(E-mail: francois.leulier@ens-lyon.fr).

Prof. em. Tore Midtvedt, MD, PhD, Department of Medical Microbial Ecology, Karolinska Institute, von Eulers Väg 5, S 171 77 Stockholm, Sweden.
(E-mail: Tore.Midtvedt@ki.se).

Giulia Nigro, PhD, Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, Unité INSERM 1202, 28 rue du Docteur Roux, 75724 PARIS Cedex 15, France.
(E-mail: giulia.nigro@pasteur.fr).

Prof. Sven Pettersson, MD, Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Box 280, S-171 77, Nobels Väg 16, S-171 77 Stockholm, Sweden.
(E-mail: Sven.Pettersson@ki.se).

Dr. rer. nat. Volker Rusch, Stiftung Old Herborn University**,** Postfach 1765**,** D-35727 Herborn-Dill**,** Germany.
(E-mail: volker.rusch@old-herborn-university.de).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 1-15 (2017).

HOST-MICROBIOME INTERACTIONS – AN EVOLUTIONARY PERSPECTIVE

THOMAS C.G. BOSCH and RENÉ AUGUSTIN

Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstr. 40, 24098 Kiel, Germany

"*NOTHING IN BIOLOGY MAKES SENSE EXCEPT IN THE LIGHT OF EVOLUTION"* (Dobzhansky, 1973)

SUMMARY

We are in the midst of a technology-driven revolution that has given us the tools to study nature. In the last 10 years, biology has made revolutionary advances from century-old debates about the relative importance of non-pathogenic bacteria. New applications of sequencing technologies are transforming our understanding of the biology of plants, animals and humans. All multicellular organisms are colonized by an assemblage of micro-organisms which, for the most part, peacefully co-exist with their hosts. Alterations to microbial communities are associated with, and likely contribute to, a number of disorders. Any organism, therefore, is considered a "metaorganism" or "holobiont".

This review examines how a growing knowledge of the animal-bacterial interactions in the phylogenetically ancient model system *Hydra* is uncovering new mechanisms to old cell biological problems that provide a foundation for understanding, preventing and in the long-term even treating diseases. We demonstrate that the epithelium is an ecosystem hosting a complex microbiome and that components of the innate immune system as well as transcriptional regulators of stem cells are involved in maintaining homeostasis between epithelia and their resident microbiota. We conclude that beneficial bacterial-host interactions should be considered an integral part of biology and that nontraditional model systems can provide a holistic understanding of the complexity of metaorganisms.

INTRODUCTION: EPITHELIA ARE ECOSYSTEMS AND HOST TO COMPLEX MICROBIAL COMMUNITIES

Our understanding of the biology of plants, animals and humans is in the midst of a major transition. Extraordinary recent progress in molecular genetics in a number of model systems, novel sequencing technologies and comparative bioinformatics is revealing details about that undermine prior conceptions, and highlight the value of an evolutionary perspective. The naïve conception that the intestinal or cutaneous epithelium is an entity, with some ectodermal and mesodermal cell types interacting with each other, is fading as

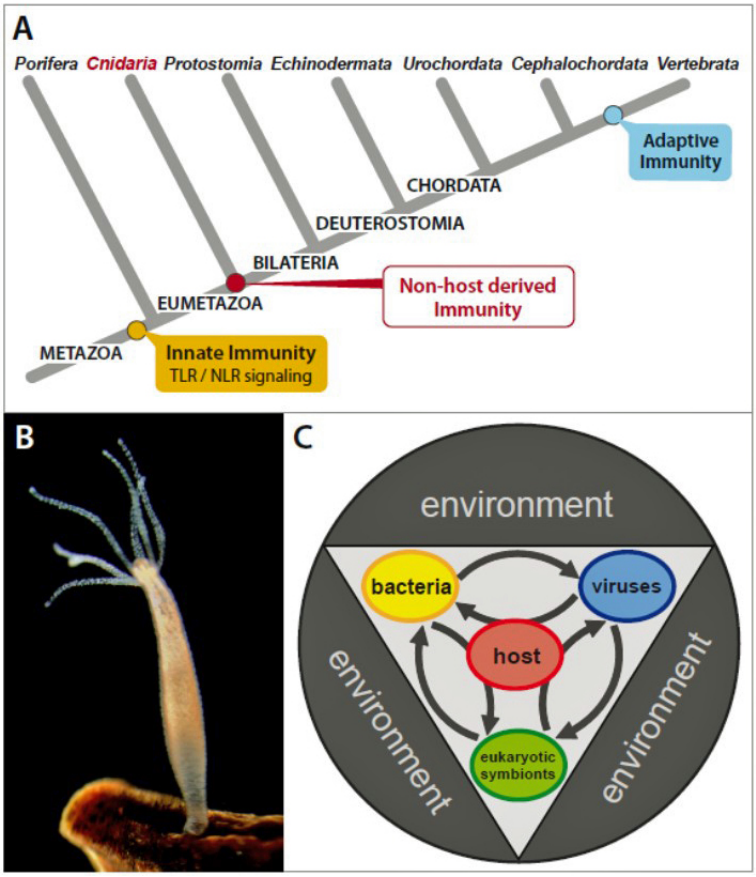

Figure 1: (A) Dendrogram showing evolutionary relationships of selected metazoans. Taxa are arranged in descending order of phylogenetic emergence relative to vertebrates. Divergence times are not to scale and tree branches are intended only to depict general relationships. (B) The freshwater polyp *Hydra vulgaris* attached to substrate (picture by S. Franzenburg). The basal metazoan has been a useful model addressing fundamental questions in immunity and host-microbe interactions in recent years. (C) Multicellular organisms are metaorganisms composed of the macroscopic host and synergistically interdependent bacteria, archaea, viruses and eukaryotic species including fungi and algal symbionts (modified from *Bosch* and *McFall-Ngai*, 2011).

the complex and dynamic nature of organisms as metaorganisms is becoming better understood. All animals, ranging from simple invertebrates to humans, are host to complex microbial communities and, therefore, must be considered a meta-organism, i.e. the macroscopic host in synergistic interdependence with bacteria, archaea, viruses, fungi, and numerous other microbial and eukaryotic species (Figure 1) (*Ley* et al., 2008; *Bosch* and *McFall-Ngai*, 2011; *Bosch* and *Miller*, 2016). These resident microbes influence fitness and thus ecologically important traits of their hosts (*McFall-Ngai* et al., 2013; *Bosch*, 2013).

Since 150 years bacteriologists, microbiologists and immunologists have focused on bacteria as pathogens. This approach has led to enormous insights in the battle between the invading harmful microbes and the host and also opened up the opportunity to develop efficient strategies to fight infections. Today we know that most bacteria are

not harmful but beneficial and are playing a key ecological role. In an updated literature survey, only about 200 of the millions of bacteria that interact with humans are regarded as emerging or reemerging pathogens (*Taylor* et al., 2001; *Woolhouse* and *Gowtage-Sequeria*, 2005). Inexpensive, high throughput sequencing has uncovered a new world of relationships between skin cells and their colonizing microbes (*Grice* and *Segre*, 2011; *Findley* et al., 2013; *Belkaid* and *Segre*, 2014; *Oh* et al., 2014). For example, using mass spectrometry and DNA sequencing, the bacteria and chemical compounds found on human skin have been sampled and mapped across the body in a series of 3-D images (*Bouslimani* et al., 2015). The chemical signature (secretome) found on the skin is thought to be unique to an individual and is distinct for certain body parts. It harbours specific combinations of bacteria and a distinct mix of molecules from foods eaten and even medicines taken. This newfound awareness of the skin as an ecosystem colonized by a diverse milieu of microorganisms presents additional layers of complexity for dermatologists and raises many questions that are being addressed by new and interdisciplinary research programs. The field of ecological evolutionary developmental biology (Eco-Evo-Devo) attempts to study and model this new view of nature by organizing concepts such as developmental symbiosis and developmental plasticity into evolutionary theory (*Gilbert* et al., 2015). "*Biology has entered a new era with the capacity to understand that an organism's genetics and fitness are inclusive of its microbiome*" (*Brucker* and *Bordenstein*, 2014).

BACTERIA MATTER: RECENT LESSONS FROM *HAEMOPHILUS*

Symbiotic microorganisms occupy a wide range of skin niches (*Grice* and *Segre*, 2011; *Findley* et al., 2013; *Belkaid* and *Segre*, 2014; *Oh* et al., 2014) and may even protect against invasion by more harmful or pathogenic organisms. Recent evidence supporting this view comes from a study focused on *Haemophilus ducreyi* (*van Rensburg* et al., 2015). This bacterium causes chancroid, a relatively common form of sexually transmitted genital ulcers that is endemic in certain parts of Africa and Asia and facilitates the transmission of HIV-1. The bacterium has also been implicated in nonsexually transmitted cutaneous ulcers in children in the tropics. Interestingly, infected individuals can either clear the infection or develop pustules that eventually form abscesses. What is the reason behind these differences? Taking advantage of a unique human skin infection model, researchers at Indiana University have found evidence to suggest that the make-up of the skin's microbiome plays a major role in whether an individual can clear the *H. ducreyi* bacterial infection without intervention (*van Rensburg* et al., 2015). The investigators compared the skin microbiome in patients who resolved their *H. ducreyi* infection to those who did not. Strikingly, pre-infection skin microbiomes of pustule formers and resolvers have distinct community structures that change in response to the progression of *H. ducreyi* infection. In people who progressed to an active infection, the microbiome was much more dispersed from the beginning of the experiment to the end than in those people who spontaneously resolved their infections. The results highlight an association be-

tween the skin's microbial inhabitants and resolution of infection. But do the bacteria that normally colonize our skin directly help to clear the pathogenic bacteria or is the microbiome only another indicator, but not the cause of bacterial infection? While we have to wait for answers to these questions, the *Haemophilus* study provides a convincing example of how the ecology of human skin can influence health and disease. Microbes therefore matter! Intriguingly, one of the abundant bacterial taxa among the infection-resolvers was *Propionibacterium acnes*, a microbe associated with skin acne. Thus it seems that under some circumstances bacterial species or strains, such as *Propionibacterium acnes*, may cause skin disease and under other conditions may guard the skin and keep it healthy. Principles of ecology appear to determine the homeostasis between skin-dwelling bacteria and their host tissue. Recent studies highlight that in addition to chancroid, the pathological outcome of many human and animal diseases is influenced by the co-existence with the residing microbial communities (*Manichanh* et al., 2006; *Oakley* et al., 2008; *Enck* et al., 2009; *Giongo* et al., 2011; *Huang* et al., 2011.

IN THE BEGINNING, NON-HOST DERIVED IMMUNITY APPEARED FOR THE FIRST TIME IN CNIDARIA …

The origin of metaorganisms represents a major evolutionary step that supported multicellular life (*Bosch* and *Miller*, 2013). Phylogenetically, stable associated microbes providing non-host derived immunity appeared for the first time in Cnidaria (Figure 1A), eumetazoan animals with a radially symmetrical, sac-like body plan. Can therefore early emerging metazoans help us to understand basic concepts that may be involved in mucosal immunity? Cnidarians such as the freshwater polyp *Hydra* are diploblastic animals consisting of an ectodermal and an endodermal epithelium (Figure 1B). While both layers are separated by an extracellular matrix (mesoglea), a true mesoderm is missing. In both layers, epithelio-muscular cells whose bodies form part of the epithelium but whose bases extend to form muscle fibres are multifunctional having both secretory and phagocytic activity. Cnidarians not only are among the earliest known phyletic lineages to form natural symbiotic relationships with bacteria and eukaryotes but also possess most of the gene families found in bilaterians and have retained many genes that have been lost in *Drosophila melanogaster* and *Caenorabditis elegans* (*Bosch* and *Miller*, 2013). For this reason, “early emerging metazoans” such as *Hydra* allow us to gain insights into the very early evolution of metaorganisms (Figure 1C).

MICROBE-EPITHELIAL INTERACTIONS IN *HYDRA*

Defining the individual microbe-host conversations in a given metaorganism (Figure 1C) is a challenging but necessary step on the path to understanding the function of the associations as a whole. Untangling the complex interactions requires simple animal models with only a few specific bacterial species. Such models can function as living test tubes and may be key to dissecting the fundamental principles that underlie all host-microbe interactions.

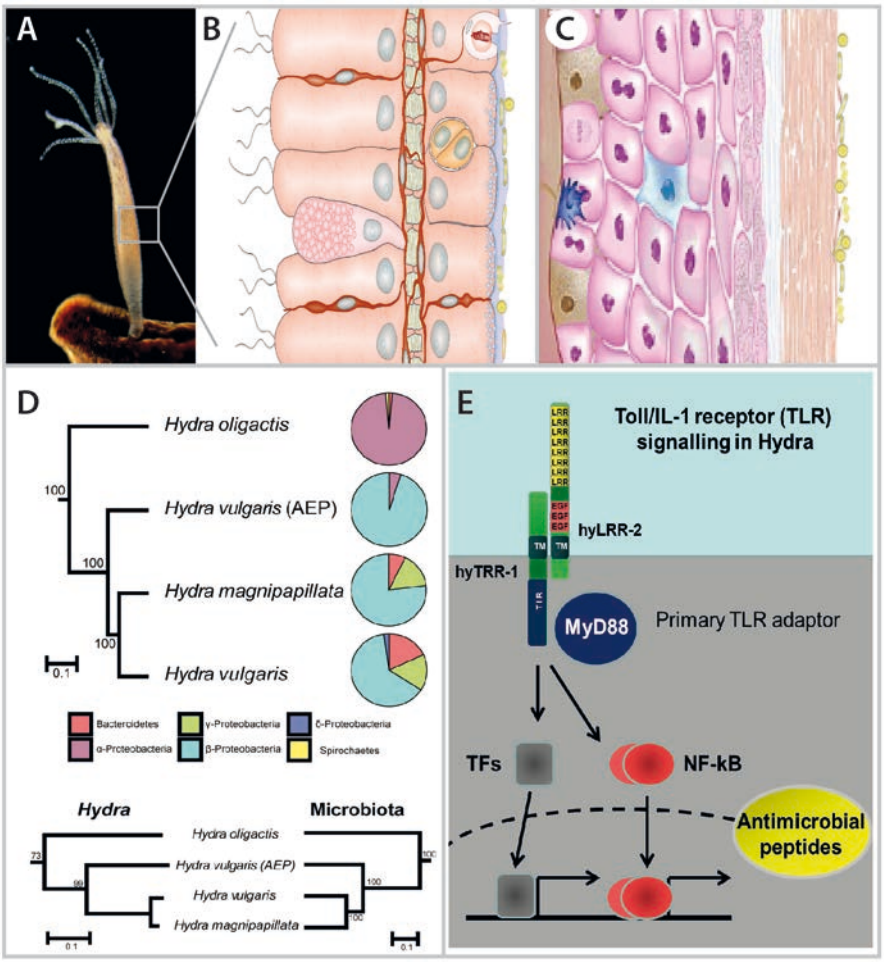

Figure 2 A) Life *Hydra vulgaris* AEP polyp (photo credit: S. Franzenburg). B) schematic representation of *Hydra* tissue including ectodermal and endodermal epithelial (with cilia) cells (orange) separated by extracellular matrix (mesoglea), gland cells (within endoderm, high vesicle content, orange), sensory and ganglion neurons (within ecto- and endoderm, red), cnidocytes (synapomorphic characteristic cell type, ectoderm, orange), glycocalyx (blue) and bacteria (yellow) (scheme credit: L. Lenk). C) Schematic representation of human skin including associated microbiota. Note structural similarities with the simple epithelium of *Hydra*. D) *Hydra* polyps are colonized by species-specific microbiota. Upper panel: Bacterial communities identified from four different *Hydra* species. Lower panel: Comparison of the phylogenetic tree from *Hydra* and the environmental cluster tree of the corresponding microbiota. E) Innate immune recognition in *Hydra* by Toll-like receptor (TLR) signalling. Recognition of bacteria is mediated by an intermolecular interaction of HyLRR-2 as receptor and HyTRR-1 as signal transducer. The HyTRR-1 molecule contains a Toll/interleukin-1 receptor (TIR) domain, a transmembrane domain, and an extracellular domain lacking any specific domain structure. The HyLRR-2 gene encodes a transmembrane protein carrying up to eight TLR-related LRR domains in its N-terminal region in addition three EGF domains. Upon activation, the receptor recruits primary adaptor molecules such as MyD88 to engage downstream signalling pathways including NF-κB. Activation of this receptor complex then triggers the innate immune response, which involves the production of antimicrobial peptides. Abbreviations: MyD88, myeloid differentiation factor 88; TM, transmembrane; TFs, transcription factors; LRR, leucine-rich repeat; EGF, epidermal growth factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells (taken from *Bosch*, 2013).

Here we introduce *Hydra* (Figure 1B; Figure 2A and B) as such a non-traditional model with one of the simplest epithelia in the animal kingdom, with only two cell layers, with few cell types derived from only three distinct stem cell lineages, and with the availability of a fully sequenced genome and numerous genomic tools including transgenesis. For analytical purposes, *Hydra* is a premier model organism, which in the laboratory is propagated and mass-cultured. The ectodermal epithelium provides a permanent protection barrier to the environment and resembles in several aspects the anatomy of the cutaneous epithelium in vertebrates (Figure 2B and C). Since the genome content (*Chapman* et al., 2010) and the ectodermal epithelial organization are remarkably similar to that of the human skin, these animals offer unique insights into the biology of a cutaneous epithelium.

MICROBIAL COLONIZATION

Bacteria are an important component of the *Hydra* metaorganism and colonize the mucus layer, which is coating the ectodermal epithelium (Figure 2B). The 36 identified bacterial phylotypes represent three different bacterial divisions and are dominated by Proteobacteria and Bacteroidetes (*Fraune* and *Bosch*, 2007; *Franzenburg* et al., 2013). Disturbances or shifts in any of these partners can compromise the health of the whole animal (*Fraune* et al., 2015). Because *Hydra* have been cultivated tens of years under standard conditions at constant temperature and identical food, it came as a surprise that examinations of the microbiota in different species kept in the laboratory for more than 20 years under controlled conditions revealed an epithelium colonized by a complex community of microbes, and that individuals from different species but cultured under identical conditions differed greatly in their microbiota. Bacteria in *Hydra*, therefore, are specific for any given species (*Franzenburg* et al., 2013). In line with this, the composition of the microbiome parallels the phylogenetic relationships of the *Hydra* species (Figure 2D). The microbiome, therefore, reflects an ancestral footprint of evolution, a pattern termed phylosymbiosis (*Brucker* and *Bordenstein*, 2013). This finding strongly indicates that distinct selective pressures are imposed on and within the *Hydra* epithelium and that the host cells actively shape the composition of its colonizing microbiota (*Bevins* and *Salzman*, 2011). Microbiota colonization can depend on the genetics of the host, and there is an intensifying interest today in resolving the relative contributions of the environment and host genes on the assembly of host-associated microbial communities. In humans, along with evidence for the influence of environmental factors, there is clear support for a host genetic component in structuring of microbial communities (*Spor* et al., 2011). In addition to candidate gene approaches, researchers have used host genome-wide genetic variation to find interactions with the microbiome. For example, in a recent genetic association study focused on psoriasis, a chronic autoimmune disease with complex genetic architecture, evidence was provided that a number of susceptibility genes are involved in innate and adaptive immunity and skin barrier functions (*Tsoi* et al., 2015). The microbiota, therefore, is a complex trait that is under strong host genetic control. The host genome may filter environmental microbes into

host tissues as a form of symbiont domestication, and reciprocally, environmental microbes may prefer to occupy specific lineages of hosts (*Brucker* and *Bordenstein*, 2012).

MICROBIAL RECOGNITION AND REGULATION

For microbial recognition, *Hydra* uses the Toll-Like Receptors (TLRs) with MyD88 as signal transducer (Figure 2E) and the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). Engagement of these receptors leads to a fast induction of protective programs. Prominent effector molecules downstream of the conserved TLR cascade are antimicrobial peptides (AMPs) (Figure 2E). AMPs in vertebrates and invertebrates act by disrupting the structure or function of the microbial cell membranes. Our work has shown that in contrast to previous assumptions these peptides are not simply killing microbes but function as host-derived regulators of the symbiotic microbiota (*Franzenburg* et al., 2013). In humans, there are three important groups of AMPs: the defensins as cationic non-glycosylated peptides containing six cysteine residues; the histatins, which are small, cationic, histidine-rich peptides present in human saliva; and cathelicidin LL-37 which is derived proteolytically from the C-terminal end of the human CAP18 protein (*De Smet* and *Contreras*, 2005). In the human genome there are at least 33 β-defensin genes. In *Hydra* the situation is much more simple. Up to now three families of potent AMPs have been identified: the hydramacin, periculin and arminin peptides. Constitutive high level expression and species-specific variability made particularly the arminin peptide family an excellent candidate for investigating the role of AMPs in shaping the host-specific microbiota. Arminin-deficient *Hydra* have a decreased ability to select suitable bacterial partners from a pool of foreign potential colonizers as they are colonized differently than control polyps, which select for bacterial types partially resembling their native microbiota (*Franzenburg* et al., 2013). We conclude from these results that AMPs are shaping the stable associated microbiota and function as host-derived regulators of microbial diversity rather than being protecting agents against pathogenic infection only.

MICROBIAL FUNCTION

Recent studies in germfree animals have shown that shifts in the microbiome can have a strong effect on host traits and could be causal in disease phenotypes (*Turnbaugh* et al., 2009). Similarly to studies in mice, we have used gnotobiotic *Hydra* to analyse the functional importance of commensal bacteria (*Fraune* et al., 2015). Bacterial colonizers in *Hydra* inhabit the outer layer of the glycocalyx and therefore, have no direct contact to the ectodermal epithelium (Figure 2B). While control cultures very rarely show signs of fungal infection, germfree *Hydra* cultures are regularly infected by fungi. Fungal hyphae are growing on the surface of germfree polyps closely attached to the ectodermal epithelium and can cause the death of the animals. Restoring the specific microbiota in gnotobiotic polyps prevents fungal

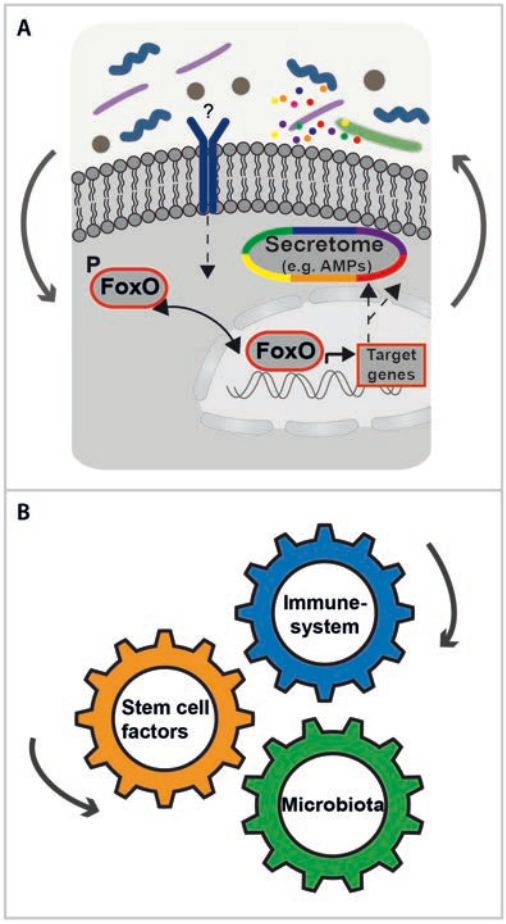

Figure 3: A) A scheme illustrating the current scenario of host gene environmental interactions. In response to changes in the microbiota FoxO activity is altered, which results in a change in expression of stem cell and immune genes, which has an impact on the maintenance of stem cell and immune system (secretome change, e.g. AMP's) and thereby on the composition of the microbiota as well as on the aging process of an organism. Young, non-aged individuals, and potentially immortal organisms such as *Hydra*, have high numbers of active stem cells and an effective immune system. Old, aged individuals, and FoxO-deficient *Hydra* are characterized by a decline in stem cell number and functionality as well as an increasingly ineffective and unspecific immune system. B) In the *Hydra* holobiont, beneficial microbes represent a major factor whose activities are linked to both tissue homeostasis, illustrated as stem cell factors, and immunity (modified from *Bosch*, 2013).

infection (*Fraune* et al., 2015). Bacteria found to significantly inhibit fungal outgrowth *in vivo* include *Acidovorax sp*., *Curvibacter sp., Pelomonas sp*. and *Undibacterium sp*.. Most importantly, none of the tested bacterial colonizers alone is able to provide full antifungal resistance. Mono-associations with distinct members of the microbiota are not efficient or fail completely to provide protection. Resistance is only achieved in polyps recolonized by a complex bacterial community. Multiple members of the microbiota act synergistically to confer resistance against the pathogenic fungus indicating that functional diversity within the commensal microbiota is central to pathogen clearance from the epithelium.

STEM CELL PROLIFERATION IS LINKED TO INNATE IMMUNITY AND MICROBIOTA COMPOSITION

As always, the unexpected is the most fascinating. In an effort to uncover the molecular logic behind *Hydra´*s unlimited life span by an unbiased transcriptome analysis, we found that the transcription factor forkhead box O (FoxO) is strongly expressed by all three stem cell lineages, whereas it is absent in differentiated cells (*Boehm* et al., 2012). By gain-of-function and loss-of-function analysis we subsequently could show that FoxO indeed is a critical component of the mechanisms controlling stem cell behaviour in immortal *Hydra*. Interestingly, silencing of FoxO activity not only affects developmental and differentiation genes but also causes changes in the expression patterns of antimicrobial peptides (AMP) which represent the immune status of *Hydra* (Figure 3A). FoxO knockdown polyps showed significant changes in expression of AMPs of the hydramacin, periculin and arminin family. In line with this, *in silico* analysis revealed multiple FoxO-binding sites on the promoter sequences of the corresponding antimicrobial peptide genes. The unexpected link between FoxO and components of the innate immune system (Figure 3) has shed at least some light on the age-old problem of how developmental pathways are linked to components of innate immunity. Taken together, it seems that beneficial microbes represent a major factor whose activities are linked to both tissue homeostasis, illustrated as stem cell factors, and immunity (Figure 3B).

THE IMMUNE SYSTEM AS HARDWARE FOR A FUNCTIONING INTERSPECIES NETWORK

Numerous observations in *Hydra* indicate that immune systems evolved as much to manage and exploit beneficial microbes as to fend off harmful ones (*Bosch*, 2015). Evidence for this view comes from the discovery that individuals from different species differ greatly in their microbiota and that individuals living in the wild are colonized by microbiota similar to that in individuals grown in the lab, pointing to the maintenance of specific microbial communities over long periods of time. As a result of the finding that interactions between animals and microbes are not specialized occurrences but rather are fundamentally important aspects of animal biology and that antimicrobial peptides and other components of the immune system are key factors for allowing the right microbes to settle and to kick the less desirable ones out, the view of the role of the immune system has changed radically in the last decade and is seen now as door-opener for symbiotic interactions (*Bosch*, 2015).

CONCLUSIONS

The increasing awareness that animals including humans exist only within a partnership with symbionts has led to two important realizations. First, the health and fitness of the skin appears fundamentally multi-organismal; and

second, an in-depth understanding of the physiology, evolution and development of organisms cannot be done on host cells only. Those unexpected insights ask for new research initiatives to systematically evaluate the critical position that microbes have in the host body. However, in spite of all these insights we have still not been able to coherently integrate the accumulated abundance of information into a truly mechanistic understanding of host-microbe interactions in a given organism. It is particularly striking that we do not even know yet what defines a healthy state of microbiota. Furthermore, how does the host control the symbiotic community composition? How stable are these host-associated species communities and how robust are they to environmental perturbations?

SIX IMPORTANT AREAS OF FUTURE RESEARCH

- Important areas of future research include developing approaches to examine at a mechanistic level how a complex microbiota interacts as a spatially and temporally dynamic network. Here, a key point is to understand to what extent the overall function of the microbiota is influenced by individual as well as synergistic contributions of community members.
- More generally, current microbiota research crucially requires us to be able to manipulate particular microbes within the community (*Bosch*, 2014). Tools to modify the presence of particular microbes are rare. Novel tools are needed to tag particular species or strains and/or identify molecules that can impact a specific taxon.
- Important areas of future research also include metagenomic screening of the associated virome, including both DNA and RNA mammalian viruses and bacteriophages, and its correlation with disease.
- What is urgently needed is to integrate information across numerous organisms and from multiple levels of organization, portraying the ecological and genetic interaction networks of entire systems, moving away from a linear cause and effect perspective. Such integrative and multi-level research approaches are required to systematically evaluate the critical role of the microbe-host assemblages as units of selection during evolution.
- With a deeper understanding of the interdependent networks and interfaces that define host-microbiota interactions, we will hopefully be able to determine whether microbial community dynamics result from disease or are implicit in instigating disease.
- This will be a key future development in guiding therapeutic strategies, including those based on engineering microbial genomes and synthetic communities. Since functional studies are crucial both for elucidating the causal mechanisms whereby microbes affect host fitness and human genetic variation impacts the microbiome, and for identifying novel treatments for inflammatory skin diseases, such as atopic dermatitis and acne vulgaris, non-traditional model systems such as *Hydra* may serve as an informative experimental tool in rethinking paradigms in medical research.

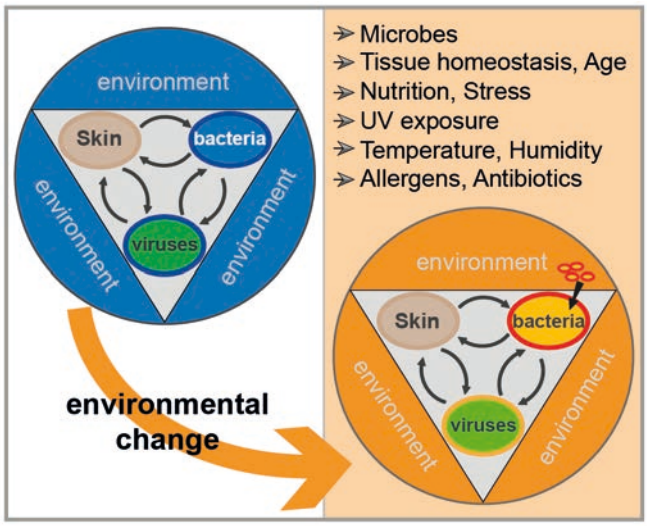

Figure 4: A model that summarize and illustrates how changes on environmental conditions influence the partner choice within the metaorganisms. The quick exchange of partners leads to a fast alteration of the functional abilities of the holobiont and allows, without immediate change of the host genome, an adaption to the changed environment.

SEEKING A HOLISTIC UNDERSTANDING OF THE CUTANEOUS EPITHELIUM

Epithelia are ecosystems carrying a myriad of microbes with them. Current efforts to understand the association between microbes and host cells treat the interacting partners as separate entities, rather than parts of one holistic system. The properties of a given symbiosis system, however, cannot be determined or explained by the specific features of its separate constituents.

We have seen above that the microbiota associated with a given cutaneous epithelium might change in response to changes in tissue homeostasis or environmental conditions. That makes it exciting to ask whether the ability of the cutaneous epithelium to adapt to stresses, to function under different environmental conditions, and resist a pathogen infection, is dependent not only on the genome of the epithelial cells, but on the genomes of its symbionts. In principle, the modularity and interoperability of the components of the metaorganism allows rapid adaptation to changing environmental conditions by altering the associated microbiota. This view was conceptualized by the "holobiont concept" (*Zilber-Rosenberg* and *Rosenberg*, 2008; *Rosenberg* et al, 2009; *Rosenberg* and *Zilber-Rosenberg*, 2013) which predicts that changes in the microbiome – from a shift in the ratio of different microbes to the acquisition of new ones – can allow the holobiont to adapt quickly to changing circumstances and even acquire new abilities during its lifetime. Depending on the variety of different niches provided by the host, which can change with developmental stage, diet or other environmental factors, a more or less diverse microbial community can be established within a given host species. The dynamic relationship between symbiotic microorganisms and environmental conditions results in the selection of the most advantageous

holobiont. A living system such as the cutaneous epithelium is nothing but a network of self-coordinating parts, which can bolster its resilience. It may be this kind of modular structure that provides the human skin with resistance against certain pathogens (such as *Haemophilus*) enabling it to fast adapting to novel environmental conditions (Figure 4).

Accordingly, and in more general terms we see host-microbe interactions as significant drivers of animal evolution and diversification (*Gilbert* et al., 2015). The forces that shaped and still are shaping the colonizing microbial composition are the focus of much current investigation, and it is evident that there are pressures exerted both by the host and the external environment to mold these ecosystems. Understanding the diversity of such genome-microbiome-environment interactions requires integrative, multidisciplinary, and modelling-based approaches (*Bosch*, 2014; *Gilbert* et al., 2015).

The newness of all of this microbiome research and the implications of these discoveries revolutionizing many aspects of biology and medicine are truly exciting. And the lessons are clear: Over decades we have learnt about the toothed wheels (Figure 3B), but we still do not understand the clock. As we recently proposed (*Bosch* and *Miller*, 2016): "*The time has come for a holistic understanding of complex life processes"*.

ACKNOWLEDGEMENTS

The work related to this review was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG), the Collaborative Research Center 1182 "Origin and Function of Metaorganisms" and the Cluster of Excellence "Inflammation at Interfaces". T.B. gratefully acknowledges support from the Canadian Institute for Advanced Research (CIFAR).

LITERATURE

Belkaid, Y. and Segre, J.A.: Dialogue between skin microbiota and immunity. Science 346, 954-959 (2014).

Bevins, C.L. and Salzman, N.H.: The potter's wheel: the host's role in sculpting its microbiota. Cell. Mol. Life Sci. 68, 3675-3685 (2011).

Boehm, A.M., Khalturin, K., Anton-Erxleben, F., Hemmrich, G., Klostermeier, U.C., Lopez-Quintero, J.A., Oberg, H.H., Puchert, M., Rosenstiel, P., Wittlieb, J., and Bosch, T.C.: FoxO is a critical regulator of stem cell maintenance in immortal Hydra. Proc. Natl. Acad. Sci. USA. 109, 19697-19702 (2012).

Bosch, T.C. and McFall-Ngai, M.J.: Meta-organisms as the new frontier. Zoology (Jena) 114, 185-190 (2011).

Bosch, T.C.: Understanding complex host-microbe interactions in Hydra. Gut Microbes 3, 345-351 (2012).

Bosch, T.C.G.: Cnidarian-microbe interactions and the origin of innate immunity in metazoans. Ann. Rev. Microbiol. 67, 499-518 (2013).

Bosch, T.C.: Rethinking the role of immunity: lessons from Hydra. Trends Immunol. 35, 495-502 (2014).

Bosch, T.C.G. and Miller, D.: The holobiont imperative. Springer, New York (2016).

Bouslimani, A., Porto, C., Rath, C.M., Wang, M., Guo, Y., Gonzalez, A., Berg-Lyon, D., Ackermann, G., Moeller Christensen, G.J., Nakatsuji, T., Zhang, L., Borkowski, A.W.,

Meehan, M.J., Dorrestein, K., Gallo, R.L., Bandeira, N., Knight, R., Alexandrov, T., and Dorrestein, P.C.: Molecular cartography of the human skin surface in 3D. Proc. Natl. Acad. Sci. USA 112, E2120-E2129 (2015).

Brucker, R.M. and Bordenstein, S.R.: Speciation by symbiosis. Trends Ecol. Evol. 27, 443-451 (2012).

Brucker, R.M. and Bordenstein, S.R.: The hologenomic basis of speciation: gut bacteria cause hybrid lethality in the genus Nasonia. Science 341, 667-669 (2013).

Brucker, R.M., and Bordenstein, S.R.: Response to Comment on "The hologenomic basis of speciation: gut bacteria cause hybrid lethality in the genus Nasonia". Science 345, 1011 (2014).

Chapman, J.A., Kirkness, E.F., Simakov, O., Hampson, S.E., Mitros, T., Weinmaier, T., Rattei, T., Balasubramanian, P.G., Borman, J., Busam, D., Disbennett, K., Pfannkoch, C., Sumin, N., Sutton, G.G., Viswanathan, L.D., Walenz, B., Goodstein, D.M., Hellsten, U., Kawashima, T., Prochnik, S.E., Putnam, N.H., Shu, S., Blumberg, B., Dana, C.E., Gee, L., Kibler, D.F., Law, L., Lindgens, D., Martinez, D.E., Peng, J., Wigge, P.A., Bertulat, B., Guder, C., Nakamura, Y., Ozbek, S., Watanabe, H., Khalturin, K., Hemmrich, G., Franke, A., Augustin, R., Fraune, S., Hayakawa, E., Hayakawa, S., Hirose, M., Hwang, J.S., Ikeo, K., Nishimiya-Fujisawa, C., Ogura, A., Takahashi, T., Steinmetz, P.R., Zhang, X., Aufschnaiter, R., Eder, M.K., Gorny, A.K., Salvenmoser, W., Heimberg, A.M., Wheeler, B.M., Peterson, K.J., Böttger, A., Tischler, P., Wolf, A., Gojobori, T., Remington, K.A., Strausberg. R.L., Venter, J.C., Technau, U., Hobmayer, B., Bosch, T.C., Holstein, T.W., Fujisawa, T., Bode, H.R., David, C.N., Rokhsar, D.S., and Steele, .RE.: The dynamic genome of Hydra. Nature 464, 592-596 (2010).

De Smet, K. and Contreras, R.: Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol. Lett. 27, 1337-1347 (2005).

Dobzhansky, T.: Nothing in biology makes sense except in light of evolution. Am. Biol. Teach. 35, 125-129 (1973).

Enck, P., Zimmermann, K., Rusch, K., Schwiertz, A., Klosterhalfen, S., and Frick, J.S.: The effects of ageing on the colonic bacterial microflora in adults. Z. Gastroenterol. 47, 653-658 (2009).

Findley, K., Oh, J., Yang, J., Conlan, S., Deming, C., Meyer, J.A., Schoenfeld, D., Nomicos, E., Park, M., NIH Intramural Sequencing Center Comparative Sequencing Program, Kong, H.H., and Segre, J.A.: Topographic diversity of fungal and bacterial communities in human skin. Nature 498, 367-370 (2013).

Gilbert, S.F., Bosch, T.C., Ledón-Rettig, C.: Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. Nat. Rev. Genet. 16, 611-622 (2015).

Giongo, A., Gano, K.A., Crabb, D.B., Mukherjee, N., Novelo, L.L., Casella, G., Drew, J.C., Ilonen, J., Knip, M., Hyöty, H., Veijola, R., Simell, T., Simell, O., Neu, J., Wasserfall, C.H., Schatz, D., Atkinson, M.A., and Triplett, E.W.: Toward defining the autoimmune microbiome for type 1 diabetes. ISME J. 5, 82-91 (2011).

Grice, E.A. and Segre, J.A.: The skin microbiome. Nat. Rev. Microbiol. 9, 244-253 (2011).

Huang, Y.J., Nelson, C.E., Brodie, E.L., Desantis, T.Z., Baek, M.S., Liu, J., Woyke, T., Allgaier, M., Bristow, J., Wiener-Kronish, J.P., Sutherland, E.R., King, T.S., Icitovic, N., Martin, R.J., Calhoun, W.J., Castro, M., Denlinger, L.C., Dimango, E., Kraft, M., Peters, S.P., Wasserman, S.I., Wechsler, M.E., Boushey, H.A., Lynch, S.V., National Heart, Lung, and Blood Institute's Asthma Clinical Research Network: Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. J. Allergy Clin. Immunol. 127, 372-381 (2011).

Franzenburg, S., Walter, J., Künzel, S., Wang, J., Baines, J.F., Bosch, T.C., and Fraune, S.: Distinct antimicrobial peptide expression

determines host species-specific bacterial associations. Proc. Natl. Acad. Sci. USA 110, E3730-E3738 (2013).

Fraune, S., and Bosch, T.C.: Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. Proc. Natl. Acad. Sci. USA 104, 13146-13151 (2007).

Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., Willoweit-Ohl, D., and Bosch, T.C.: Bacteria-bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance. ISME J. 9, 1543-1556 (2015).

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I.: Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776-788 (2008).

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., Roca, J., and Dore, J.: Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55, 205-211 (2006).

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J.G., Tautz, D., and Wernegreen, J.J.: Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci, USA 110, 3229-3236 (2013).

Oakley, B.B., Fiedler, T.L., Marrazzo, J.M. and Fredricks, D.N.: Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. Appl. Environ. Microbiol. 74, 4898-4909 (2008).

Oh, J., Byrd, A.L., Deming, C., Conlan, S. NISC Comparative Sequencing Program, Kong, H.H., Segre, J.A.: Biogeography and individuality shape function in the human skin metagenome. Nature 514, 59-64 (2014).

Rosenberg, E., Sharon, G., and Zilber-Rosenberg, I.: The hologenome theory of evolution contains Lamarckian aspects within a Darwinian framework. Environ. Microbiol. 11, 2959-2962 (2009).

Rosenberg, E., and Zilber-Rosenberg, I.: The hologenome concept: human, animal and plant microbiota. Springer International Publishing, Switzerland (2013).

Spor, A., Koren, O., and Ley, R.: Unravelling the effects of the environment and host genotype on the gut microbiome. Nat. Rev. Microbiol. 9, 279-290 (2011).

Taylor, L.H., Latham, S.M., and Woolhouse, M.E.: Risk factors for human disease emergence. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 356, 983-989 (2001).

Tsoi, L.C., Spain, S.L., Ellinghaus, E., Stuart, P.E., Capon, F., Knight, J., Tejasvi, T., Kang, H.M., Allen, M.H., Lambert, S., Stoll, S.W., Weidinger, S., Gudjonsson, J.E., Koks, S., Kingo, K., Esko, T., Das, S., Metspalu, A., Weichenthal, M., Enerback, C., Krueger, G.G., Voorhees, J.J., Chandran, V., Rosen, C.F., Rahman, P., Gladman, D.D., Reis, A., Nair, R.P., Franke, A., Barker, J.N., Abecasis, G.R., Trembath, R.C., and Elder, J.T.: Enhanced meta-analysis and replication studies identify five new psoriasis susceptibility loci. Nat. Commun. 6, 7001 (2015).

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., and Gordon, J.I.: A core gut microbiome in obese and lean twins. Nature 457, 480-484 (2009).

van Rensburg, J.J., Lin, H., Gao, X., Toh, E., Fortney, K.R., Ellinger, S., Zwickl, B., Janowicz, D.M., Katz, B.P., Nelson, D.E., Dong, Q., and Spinola, S.M.: The human skin microbiome associates with the outcome of and is influenced by bacterial infection. mBio. 6, e01315-15 (2015).

Waldor, M.K., Tyson, G., Borenstein, E., Ochman, H., Moeller, A., Finlay, B.B., Kong, H.H., Gordon, J.I., Nelson, K.E., Dabbagh,

K., and Smith, H.: Where next for microbiome research? PLoS Biol. 13, e1002050 (2015).

Woolhouse, M.E. and Gowtage-Sequeria, S.: Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. 11, 1842-1847 (2005).

Zilber-Rosenberg, I. and Rosenberg, E.: Role of microorganims in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol. Rev. 32, 723-735 (2008).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 17-31 (2017).

STUDYING HOST-MICROBIOTA MUTUALISM USING GNOTOBIOTIC DROSOPHILA

DALI MA, GILLES STORELLI, MÉLANIE MITCHELL, and FRANÇOIS LEULIER

Institut de Génomique Fonctionnelle de Lyon (IGFL),
Ecole Normale Supérieure de Lyon, CNRS UMR 5242,
Université Claude Bernard Lyon 1,
69364 Lyon Cedex 07, France

SUMMARY

The complex interaction between the metazoan host and its commensal gut microbiota is one of the essential features of symbiosis in the animal kingdom. As there is a burgeoning interest to decipher the molecular dialogue that shapes host-microbiota mutualism, the use of gnotobiotic model organism becomes an imperative approach to unambiguously parse the specific contributions to such interaction from the microbiome. In this review, we focus on several remarkable gnotobiotic studies in *Drosophila* that functionally depicted how the gut microbes can alter host physiology and behaviour through transcriptomic regulation, hormonal control and diet modification. These results in concert illustrate that the gnotobiotic flies mono- or poly-associated with members of its gut microbiota deliver a versatile and powerful model that is amenable to different types of studies ranging from classic genetics to large-scale systems approaches.

INTRODUCTION

In 1883, Louis Pasteur expressed his wish to raise a "microbially deprived" young animal on "pure" food from birth, and postulated that "without any preconceived notion…. life under such condition… shall become impossible" (*Pasteur*, 1885). Nearly 30 years later, Eugene Wollman at the Pasteur institute in Paris successfully cultured the first germfree common blowflies (*Calliphora vomitoria*) and observed that except for certain minor growth delay, the adult flies appeared perfectly normal (*Wollman*, 1911). At first, Wollman's experiment seemed to have put an end to Pasteur's claim, yet in truth it was only the beginning. Throughout his productive career as a microbiologist, Wollman probably did not realize that his germfree blowflies spawned an entire field of animal physiology based on host-microbe interactions; and only when a germfree life was made possible, the concept of "gnotobiology" could spring to life. In the past century, Pasteur's musing on what life would be like without its resident microbes gradually transformed to a quest to understand how the eukaryotic hosts and their bacterial partners orchestrate the symphony of life, and how such interactions probably profoundly changed the course of our evolutionary history (*McFall-Ngai* et al., 2013).

Microbes occupy every possible ecological niche on earth. A set of particular niches comprise the various

internal epithelia of the metazoan hosts, who, through eons of evolution, have forged complex and intricate relationships with this rich and diverse microbial community, called the "microbiota" (*McFall-Ngai* et al., 2013; *Douglas*, 2014). A human host carries on his body far more microorganisms than his own cells, and these invisible dwellers constitute 1-3% of his body mass (*Human Microbiome Project*, 2012; *Sommer* and *Backhed*, 2013). The human gut alone harbours approximately 500 to 1,000 bacterial species (*Eckburg* et al., 2005), and represents the largest mucosal surface where the exchanges between the host and the microbiota take place. In the last decades, many studies together generated a systematic understanding of how the gut microbiota and its diverse gene repertoire, called the "microbiome", can configure the fitness parameters of the host; a healthy microbiota can expand the host's metabolic potential, fortify its immune system, promote healthy aging and even dictate its emotional and psychological well-being (reviewed in: *Grenham* et al., 2011; *Clemente* et al., 2012; *Sommer* and *Backhed*, 2013; *Kaiko* and *Stappenbeck*, 2014; and *Sharon* et al., 2014). However, as the community structure and activities of the gut microbiota are extremely sensitive to fluctuations in the environment, perturbations to the microbiota pose significant risks to the host (reviewed in: *O'Hara* and *Shanahan*, 2006; and *Gibson* et al., 2014). Subtle changes in host immunity, diet or xenobiotic concentration can disrupt the balance in the gut microbial community, which consequently compromises host fitness. In mammals, microbiome imbalance, or dysbiosis, positively correlates to the onset of obesity, diabetes, colon cancer (reviewed in: *Tremaroli* and *Backhed*, 2012; *Karlsson* et al., 2013; and *Irrazabal* et al., 2014) and human psychiatric disorders such as schizophrenia and autism (reviewed in *Fond* et al., 2014).

Currently, a large amount of research on host-microbiota mutualism employs vertebrate models, yet the high complexity of the microbial composition in the mammalian gut, the difficulty to culture most of these microbial species, and the cost of raising these animals in a strictly sterile environment pose a considerable obstacle. Therefore, to delve deeper into the molecular interplay between the host genome and the microbiome and the environmental contributions to such interplay, a more genetically tractable model organism with simpler and even defined microbiota is an attractive option. *Drosophila melanogaster* fits these criteria. First of all, the intestinal tract of the fruit fly is anatomically and physiologically similar to the mammalian gut (*Lemaitre* and *Miguel-Aliaga*, 2013), yet the microbial composition is rather simple: throughout the larval and adult life, the fly gut hosts five to twenty aero-tolerant commensal species, all of which are readily cultured in the laboratory (*Broderick* and *Lemaitre*, 2012; *Erkosar* et al., 2013). Two families of bacteria: Acetobacteraceae and Lactobacillaceae, dominate the community (*Chandler* et al., 2011; *Shin* et al., 2011; *Storelli* et al., 2011; *Wong* et al., 2011; *Ridley* et al., 2012; *Chaston* et al., 2014). However, the fly gut microbiota is transient in nature and requires constant replenishment, thus the community structure and bacterial load fluctuate highly as the flies develop and age (*Blum* et al., 2013; *Broderick* et al., 2014; *Erkosar* and *Leulier*, 2014). Such inconstancy makes it difficult to clearly pinpoint the bacterial genetic factors contributing to host physiology. Therefore, the use of gnotobiotic fly models, in combination with classic genetic approaches and next-generation sequencing, proves to

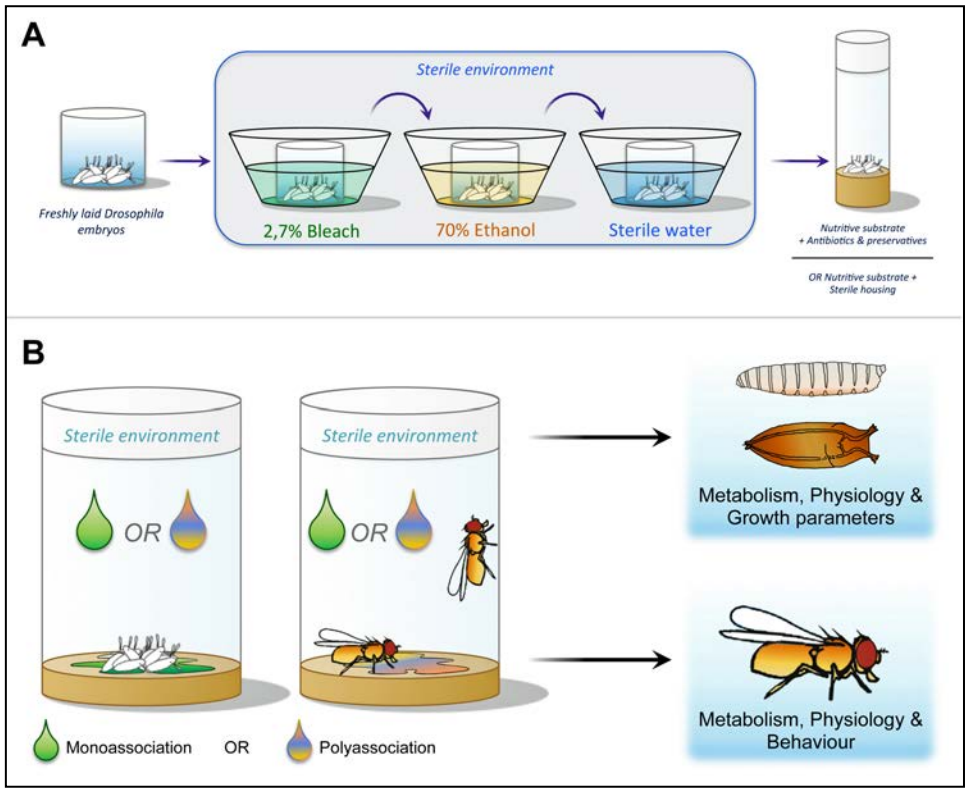

Figure 1: Building a gnotobiotic *Drosophila* model to study host-microbiota mutualism.
A. To obtain germfree flies, freshly laid eggs are harvested in large-scale and washed in succession with bleach, ethanol and sterile water. To maintain axeny, the dechorionated eggs are then grown in the presence of antibiotics and preservatives or in a sterile environment.
B. To study the specific contribution of the microbiome to the different aspects of host physiology, ex-axenic eggs or adults are mono-associated with a single gut commensal species (green drop) or poly-associated with a defined set of gut commensal bacteria (blue and yellow coloured drop). Such gnotobiotic flies have been used to study the impact of specific commensals on host juvenile growth, developmental timing, metabolic homeostasis and adult behaviour.

be the new and effective means to study intestinal mutualism with added advantage, because it enables the investigators to inoculate the germfree subjects with various bacterial strains of predefined quantity and composition – such of any member of the fly microbiota. In this setting, the researchers not only can rigorously monitor the phenotypic changes in different aspects of host physiology, but also can robustly correlate and even attribute particular changes to the specific functions from the microbiome, as the genomes of many gut microbiota species are being rapidly sequenced and annotated (*Human Microbiome Project*, 2012). Moreover, except for *Acetobacter*, which is mostly found in insects (*Crotti* et al., 2009; *Chouaia* et al., 2014), *Lactobacillus* species are commensal to mammals (*Reuter*, 2001; *Rastall*, 2004 ; *Walter* et al., 2011). Therefore, the results from such gnotobiotic fly studies can be readily translated to mammalian studies. *Drosophila* models were first used to dissect the genetic networks governing host/pathogen interaction

(*Buchon* et al., 2014). With the same approach, pioneering studies have shown promising results to identify and functionally characterize the genetic components of the molecular crosstalk between *Drosophila* and its commensal bacteria. In this review, we discuss the findings from the studies using gnotobiotic fly models to unravel the impact of the members of gut microbiota on host metabolism, physiology and behaviour (Figure 1).

THE MAKING OF THE GNOTOBIOTIC FLIES

As mentioned before, in the early 1910s, Eugene Wollman and his colleagues at the Pasteur institute were among the first to raise germfree animals such as common blowflies, tadpoles and guinea pigs. Wollman made the first germfree common blowflies by treating the egg surface with diluted hydrogen peroxide and raising the larvae on sterilized meat substrate (*Wollman*, 1911). Interestingly, Wollman observed that the germfree larvae reached normal body size, but at a slower rate. Moreover, these flies seemed slower in movement and less interested in foraging. Therefore, even though the "microbially deprived" life was indeed possible in a sterile environment, the difference between such a life and it's conventionally reared (CR) siblings was already observable to the naked eye. In the next few decades, *Drosophila melanogaster* was attaining a more and more prominent status as a model genetic organism. As a result, in the 1950s and '60s, different methods were developed to sterilize *Drosophila* eggs on a large-scale and keeping axenic fly stocks turned into a routine laboratory practice.

In 1969, Marion Bakula developed the first monoxenic *Drosophila* model by associating bleached fly eggs with either "native" or "foreign" bacteria strains (*E. coli*) (*Bakula*, 1969). In her study, only the "native" bacteria isolated from the fly gut persisted throughout larval development in the fly host, who pupariated at a slightly faster pace than the axenic controls. This is also the first gnotobiotic model to demonstrate that the essential mode of microbial transmission in fruit flies is through larvae ingestion of the contaminated chorion. Therefore, thorough dechorionation of the eggs can effectively render a fly stock germfree. In the next several decades, after trying different sterilizing agents such as antiformin and formalin (*Begg* and *Sang*, 1950) researchers found that the treatment with common household bleach (diluted sodium hypochloride solution) in combination with ethanol wash is the safe, simple, rapid and effective way to dechorionate the embryo and rid the surface of bacterial "contaminants". However, bleaching alone cannot eliminate intracellular endosymbionts such as *Wolbachia*, the most widespread insect symbiont whose relationship with the host ranges from parasitism to mutualism. Depending on the context, the presence of *Wolbachia* is known to affect reproductive success, enhance insulin signalling and boost host defence (*Ikeya* et al., 2009; *Gronke* et al., 2010; *Ringo* et al., 2011; *Hamilton* and *Perlman*, 2013). Therefore, to obtain a "true" germfree or gut-commensal specific phenotype unadulterated by *Wolbachia*, different laboratories have adopted various protocols to maintain germfree stocks, either by combining bleaching with rearing flies on food containing a mixture of antibiotics, or by one-time treatment of bleach and the subsequent maintenance of the flies in

a sterile environment (Figure 1A). Of note, bleaching and/or antibiotic treatment can lower fly viability and fecundity and have certain unintended negative cellular and systemic effects on the host (*Ridley* et al., 2013). Therefore, the studies using germfree flies mandate careful and thorough controls. In the following sections, we review a few seminal gnotobiotic *Drosophila* studies that have uncovered important molecular mechanisms governing host-microbiota interaction.

THE STUDY OF HOST PHYSIOLOGY USING A GNOTOBIOTIC FLY MODEL

A gnotobiotic fly model with classic genetics approach

That the germfree flies develop and grow at a slower pace is an old observation that has held true since Wollman's time. For example, in Baluka's monoxenic culture, the native bacterial isolates from the *Drosophila* gut: Stock 13, a *Brevibacterium* variant, accelerated pupariation compared to the axenic stock (*Bakula*, 1969). This observation has now been further characterized in greater detail. On a "standard" laboratory diet, the pupariation and adult eclosion rate of the axenic flies are delayed by one day compared to their CR siblings (*Shin* et al., 2011; *Wong* et al., 2014). However, this delay becomes striking when the axenic flies are presented with nutritive challenges. Particularly, when raised on a diet where the yeast content was below 0.1%, or was completely replaced by casamino acids, the germfree flies die (*Shin* et al., 2011). This observation suggests that an intact gut microbiota provides life-sustaining factors for the host experiencing severe nutritive duress. Next, when fed on a diet with low yeast content, germfree flies pupariate six days later than the CR flies (*Storelli* et al., 2011). Therefore, the gut microbioa can also override the developmental delay to potentiate growth in suboptimal nutritive environment. Importantly, these two studies also demonstrated that inoculating the axenic fly embryos with one or several defined gut commensal species, such as *Lactobacillus plantarum (L. plantarum)* or *Acetobacter pomorum (A. pomorum),* can recapitulate the growth benefits conferred by the entire gut microbiota. Moreover, only certain strains of *L. plantarum* sustain growth on a low-yeast diet: several other isolates from the fly origin were unable to promote host growth even though they could colonize the larval gut and the fly food just as efficiently as the beneficial strains (*Storelli* et al., 2011). This observation unequivocally illustrates that the gut microbiota promotes growth by not just serving as a food source, but through complex molecular and biochemical interactions with the host.

How then, does the gut microbiota promote host growth? First of all, like for many metazoan species, the source of the fly gut bacteria comes from contaminated food (*Broderick* and *Lemaitre*, 2012; *Erkosar* and *Leulier*, 2014), and naturally, some of the primary functions of the gut bacteria are to enhance digestion and expand the host's metabolic potential. The additional enzymatic activities from the bacterial origin help break down the specific nutritive substrates that are otherwise indigestible for the host, who

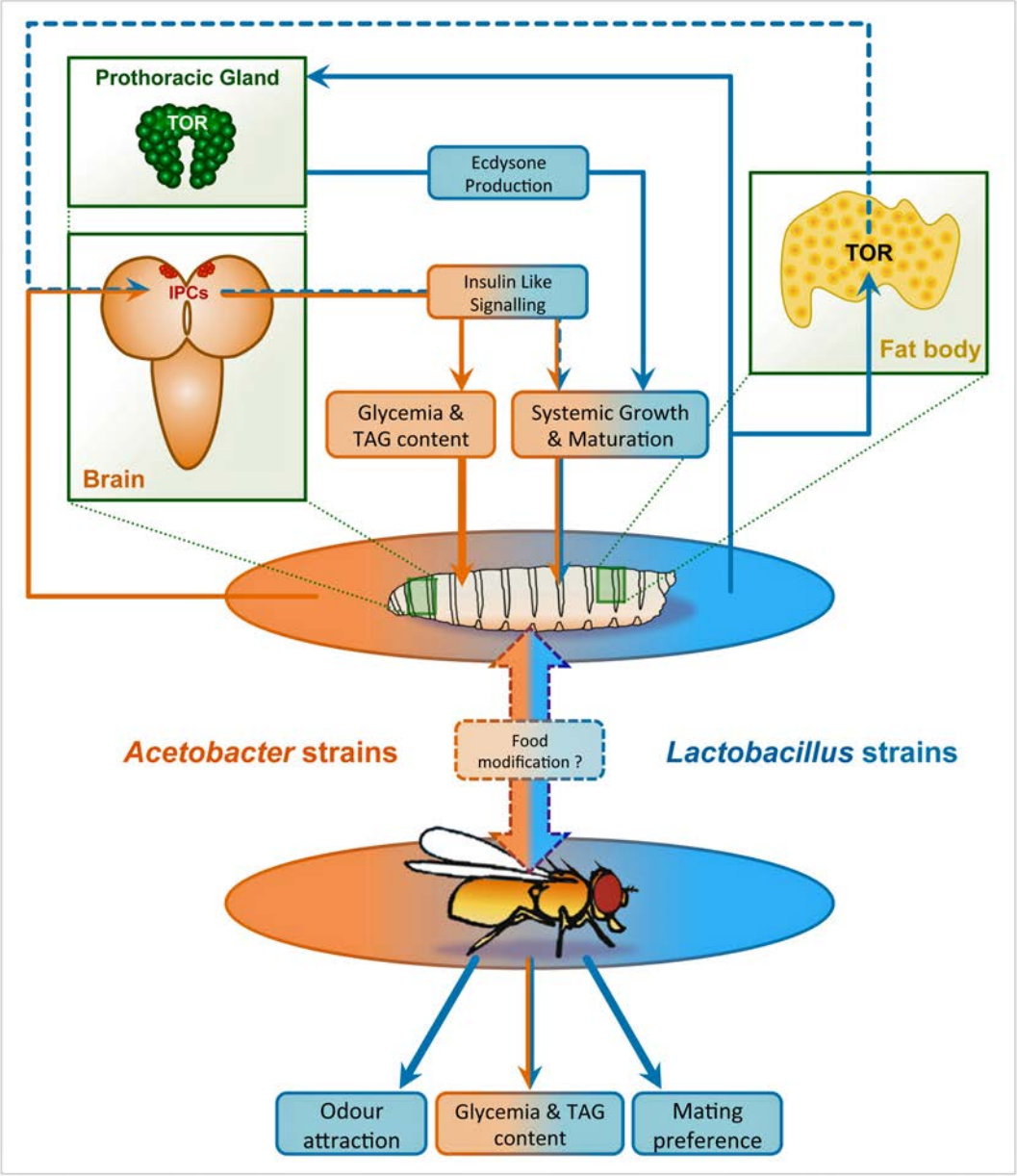

Figure 2: The host physiological and behavioural responses to the addition of different gut commensal strains.
Gnotobiotic studies have depicted the effect of *Acetobacter* and *Lactobacillus* strains on systemic growth, metabolic homeostasis and adult behaviour. Specifically, in the presence of nutritive challenge, *A. pomorum* (orange) regulates host insulin signalling in the insulin producing cells (IPCs) and thus promotes larval growth and maturation, whereas *L. plantarum* (blue) interacts with host TOR (target of rapamycin) pathway in the fat body and the prothoracic gland to control ecdysone production and affects insulin signalling directly or indirectly (dotted blue line). During the adult stage, both *Acetobacter* and *Lactobacillus* strains regulate host triacylglyceride (TAG) and circulating glucose levels, but only *Lactobacillus* strains have been shown to impact host behaviours such as mating preference and odour attraction to food. The effect of the gut commensals represented by *Acetobacter* and *Lactobacillus* can be direct or via modifying the nutritional substrates.

can in turn harvest energy from these food substrates and extract necessary metabolic building blocks for various biological processes (*Sommer* and *Backhed*, 2013). In addition, essential micronutrients derived from bacterial metabolism such as vitamins and short chain fatty acids directly fuel the host's metabolism (*Natarajan* and *Pluznick*, 2014). Indeed, two recent studies found that fortifying the food fed to the germfree flies with B vitamins phenocopies the effect of the presence of the gut bacteria to a large extent, indicating that the gut microbiota accomplishes metabolic sparing of the B vitamins for the host through a yet unknown mechanism (*Fridmann-Sirkis* et al., 2014; *Wong* et al., 2014).

However, the growth benefits from the gut microbiota are probably beyond vitamin B provision. To identify the microbial factors that can rescue host lethality on the casein diet, Shin et al. conducted a random mutagenesis in *A. pomorum* and isolated strains that restored ex-germfree larval survival on casamino acid diet but led to delayed pupariation when compared to animals mono-associated with the wild-type *A. pomorum*. Several such mutations affect Pyrroloquinoline quinone-dependant alcohol dehydrogenase (Pqq-adh), an enzyme involved in the ethanol respiratory chain, and whose end product is acetic acid. Although *Pqq-adh* mutant bacterial strains were impaired in their production of acetic acid, supplementation of casamino acid diet with acetic acid alone failed to rescue germfree larval lethality. However, concomitant association with *Pqq-adh* mutant *A. pomorum* strains and supplementation with acetic acid completely rescued larval developmental timing. Therefore, upon severe nutritive challenge, the addition of *A. pomorum* first and foremost restores the viability of the fly host, and then the intact activity of the bacterial ethanol respiratory chain promotes host growth and maturation. Based on this result, it is likely that the molecular mechanisms that sustain larval life and promote growth are separable.

Genetic factors from the host

What are the host factors responding to the beneficial growth promotion effect of the microbiota in the presence of nutritional challenges? The studies of *Shin* et al. (2011) and *Storelli* et al. (2011) demonstrate that the addition of *A. pomorum* or *L. plantarum* can accelerate growth and maturation by modulating host systemic hormonal signalling. In the Shin et al study, larvae mono-associated with the *Pqq-adh* mutant strain of *A. pomorum* survived to adulthood, but displayed metabolic features reminiscent of defective insulin/insulin like growth factor (IIS) signalling, such as low body weight, retarded growth, elevated haemolymph glucose and trehalose levels, and higher level of triacylglyeride (TAG), the main form of stored lipids. At the molecular level, in the fat body of the flies mono-associated with mutant *Pqq-adh A. pomorum* strains on the casamino diet, membrane activation of PI3K and cytoplasmic retention of dFOXO were abolished, and the expression of Insulin-Like peptides (Dilps) such as Dilp3 and 5 was reduced in the larval brain. Most importantly, the ectopic expression of Dilp2 largely rescued both the defective IIS phenotype and the molecular signatures associated with such defects in flies mono-associated with mutant strain of *A. pomorum*. Therefore, *A. pomorum*, partly via its *ppq-adh* activity, regulates IIS to maintain the host's metabolic homeostasis (Figure 2). Similarly, on a low-yeast diet, mono-

association with *L. plantarum* lowered the expression of insulin receptor, a negative readout of pathway activity, suggesting that the presence of *L. plantarum* also enhances insulin signalling (*Storelli* et al., 2011). Moreover, *L. plantarum* reduced the juvenile growth period through TOR signalling: dampening TOR activity in the fat body – the functional analogue of the mammalian liver – and the prothoracic gland compromised the *L. plantarum* growth promoting effect as measured by adult emergence (Figure 2). TOR is the host nutrient-sensitive signalling pathway devoted to balance organismal growth and maturation in a nutrient-dependent manner (*Hietakangas* and *Cohen*, 2009; *Danielsen* et al., 2013). In the developing larvae, TOR activity in the prothoracic gland directly controls ecdysone production, which in turn affects the parameters of systemic growth via IIS. As TOR responds to the circulating levels of different micronutrients in the haemolymph, such as branched-chain amino acids, *L. plantarum* may act upstream of TOR in several ways. First, *L. plantarum* can directly regulate TOR activity by making certain metabolites or other biochemical pathway intermediates or/and end products. Secondly, *L. plantarum* can either modify the diet or boost the host's digestive capacity to enhance nutrient assimilation, which then indirectly activates TOR pathway. Therefore, how *L. plantarum* promotes host juvenile growth is to yet be studied in detail.

Now two groups have demonstrated that specific strains from both *Acetobacter* and *Lactobacillus* families can promote juvenile growth upon nutritive challenge (*Shin* et al., 2011; *Storelli* et al., 2011). What effect does the combined action of *Acetobacter* and *Lactobacillus* have on the host? To study how these two commensal bacteria interact in the host and how such interactions impact adult host physiology, a study by Newell and Douglas compared differences in circulating glucose levels, TAG contents and adult body weight between axenic flies and ex-germfree flies associated with a single or different combinations of the five fly commensal species (*Newell* and *Douglas*, 2014). Specifically, using a set of defined microbiomes with up to five commensal species (*A. pomorum*, *A. tropicalis*, *L. plantarum*, *L. brevis*, and *L. fructivorans*), the authors inoculated the germfree flies with one or different combinations of these strains and found that all these combinations lowered circulating glucose concentrations in comparison to axenic flies. However, in terms of lowering host TAG levels, these different combinations of bacteria worked at different efficiency: the *Lactobacillus* species can lower TAG level moderately; *Acetobacter* did so more effectively than *Lactobacillus*, but not as effectively as the five species co-inoculation, which was the only treatment that recapitulated the benefits of the conventional commensal flora. Interestingly, one specific co-inoculation, with *Acetobacter tropicalis* and *Lactobacillus brevis*, was particularly potent in that it lowered host TAGs more than in animals poly-associated with the five commensal species. Other forms of co-associations with two bacterial representatives from the *Lactobacillus* and *Acetobacter* genera failed to reproduce the phenotype. These results indicate that *Acetobacter* and *Lactobacillus* strains can act in synergy, but not consistently. A plausible explanation for this puzzling phenomenon is that the content of the microbiome, rather than the taxonomic combination, determines the TAG content of the host (see below).

Gnotobiotic model with systems approach

Large-scale identification of bacterial genetic determinants

Using classic genetic analysis on a mono-association fly model, *Shin* et al. (2011) and *Storelli* et al. (2011) identified the entry points to further dissect the molecular dialogue between the host and the gut microbiota that alters host physiological traits. Gnotobiotic models are now deployed for large-scale search to fit the same purpose. In a metagenomic study, Chaston and colleagues first gathered a collection of 41 fully sequenced bacteria strains broadly encompassing different *Acetobacter* and *Lactobacillus* genus. Raised in mono-association with each of the 41 strains, the fly hosts showed a spectrum of different responses in terms of pupariation timing and adult TAG content. Based on the comparison of the amplitude of the mono-association effect on these two parameters, the authors undertook a metagenome-wide association study (MGWAS) that effectively correlates bacterial genetic determinants to the magnitude of changes in developmental timing and TAG content (*Chaston* et al., 2014). Remarkably, the MGWAS based on developmental timing first yielded clusters of genes operating in the cellular respiratory chain, including the PQQ enzyme that converts sugar and alcohol substrates to acetic acid. This result corroborates the finding from the transposon screen in *A. pomorum* by *Shin* et al. (2011). Interestingly, they recovered the *Pqq* mutant bacteria on a casein-only fly medium that causes lethality in germfree flies, whereas the MGWAS was conducted on standard laboratory fly food, where the developmental delay in germfree flies was subtle. At a glance, it is a bit surprising that both studies uncovered the same bacterial factor based on host developmental timing, a trait that varies drastically in the two experimental setups. It provocatively suggests there is a robust and canonical host interaction with bacterial ethanol respiratory chain products that cannot be masked by different host nutritional backgrounds. Such response has been shown to involve host insulin signalling. How such robust interaction is maintained in a different nutritional background is an extremely interesting topic to explore. Moreover, in the study by *Chaston* et al. (2014), the clusters of genes that correlated to lower host TAG content are known to regulate redox sensing and glucose oxidation, such as glucose dehydrogenase (GDH), gluconate-2 dehydrogenase (GnDH) and a single domain oxidoreductase (SDR). Importantly, introducing these candidate genes into selected *Acetobacter* strains that lack these enzymes conferred the ability to the bacteria to reduce host TAG level. Furthermore, the authors observed that ectopic expression of GDH and GnDH concomitantly lowered glucose content in the media where the gnotobiotic flies were raised. These results strongly suggest that the gut microbiota can modulate host lipid storage and nutritional homeostasis through altering the nutrient composition of the food. Another intriguing observation from the study is that the clustering of bacterial strains based on the effect on host developmental timing and TAG level is largely unrestricted to the taxonomic structure of the bacteria. Hence, the collective genetic composition of the gut microbiome once again proves to be a more faithful predictor of host response than taxonomic classification. Now looking back, the finding by *Chaston* et al. (2014) probably also partially explains why *Newell* and *Douglas* (2014) observed inconsistent TAG lowering ef-

fect in flies associated with different combinations of *Acetobacter* and *Lactobacillus* strains (see the previous section). Altogether, this particular study raises a few interesting issues. For example, Chaston and colleagues propose that by modifying the food, bacterial glucose metabolism impacts the adult host's capacity to store lipid (*Chaston* et al., 2014). Does this observation hold true in the developing larvae? The published studies seem to favour the likelihood, as *Shin* et al. (2011) unequivocally demonstrated that gnotobiotic larvae harbouring mutant *Pqq* mutant *Acetobacter* strain show higher circulating sugar and triglyceride as a result of the compromise in the host insulin signalling activity (Figure 2). If this is the case, do the bacteria directly elicit the host insulin response, or is such insulin response an indirect result of bacteria altering the glucose content of the food? These two possibilities are not mutually exclusive but require further detailed mechanistic studies that either tease them apart or meld them together. So far Chaston and colleagues were unable to rescue developmental delay by ectopically expressing the enzymes involved in glucose oxidation, but such negative outcome likely to imply that the interaction between host maturation and microbiota metabolism is more complex than we think.

The host's transcriptomic response to gnotobiotic association

The association with certain commensal species modulate host IIS and TOR signalling. What other kind of molecular changes take place in the host in the presence of the gut microbiota? To answer this question, several groups recently undertook microarray studies to compare the transcriptomic differences between the germfree flies and their conventionally reared siblings at different age, and demonstrated that in the fly gut, the presence of the microbiota significantly alters the expression of a core set of genes that control transcription, gut structure, immunity, metabolism, signalling and stress response (*Broderick* et al., 2014; *Guo* et al., 2014) and reviewed in *Erkosar* and *Leulier* (2014). Among these studies, Guo and colleagues extended the microarray finding and elegantly showed that in the aging fly gut, the transcription factor Foxo represses peptidoglycan recognition protein SC2 (PGRP-SC2), which subsequently leads to hyperactivation of Rel/NFkB activity that is responsible for an intestinal dysbiosis phenotype. In addition to these studies, another noteworthy microarray analysis using poly-associated gnotobiotic flies identified a short but focused list set of genes whose functions are enriched in digestion and primary metabolism. *Erkosar* et al. (2013) conducted the microarray study on exgermfree adult flies exposed to a defined set commensal bacterial strains (*A. pomorum, Commensalibacter intestini, Lactobacillus brevis and L. plantarum*). First, the polyassociation yielded certain genes that overlap with those found in the concomitant study by *Broderick* et al. (2014) using CR flies. Specifically, such polyassociation markedly up-regulates the expression of a set of digestive enzymes and other genes involved in primary metabolism. This result reflects the conventional notion that gut bacteria assist in host digestive functions to effectively extract nutrients and energy from food. Intriguingly still, half of the polyassociation upregulated genes identified by *Erkosar* et al. (2013) were also involved in response to intestinal infection, and the majority of these genes are directly or indirectly under the control of Relish, the *Drosophila* orthologue of the mammalian NFκB factor p105 (*Buchon* et al., 2009). This

result once again corroborates the study by *Broderick* et al. (2014), who also observed that more than half of the up-regulated genes in the CR fly gut changed expression pattern in *Relish* mutant flies. As Relish is essential to the interplay between host innate immunity and nutritional response, *Erkosar* et al. (2013) postulated the following scenario: the presence of commensal strains usually promotes the expression of a certain set of digestive enzymes and metabolic genes, but in the presence of an acute infection, a change in the host transcriptome is triggered, so that these microbiota-mediated metabolic genes are down-regulated to prepare for immune defence, and such change is mediated by Relish. Consistent with this hypothesis, the authors found that the expression patterns of several selected candidate genes such as *trypsin* and *Jonah* proteases are indeed down-regulated upon pathogen infection or in the genetic background where Relish activity is compromised. In summary, the finding by *Erkosar* et al. (2013) first largely recapitulates the host's transcriptomic response to gut-microbiota in CR flies, thus cementing the utility and relevance of the poly-association model. Furthermore, like in *Broderick* et al. (2014), the authors identified Relish as the central regulator of a transcriptional trade-off between metabolic response and immunity, and thus opened a new chapter for potential mechanistic studies of such switch. Altogether, the studies by *Erkosar* et al. (2013) and others were the first to demonstrate that the gut microbiota profoundly alters the host transcriptomic landscape, yet we know little how the bacteria mechanistically effect these changes. Secondly, these studies also provide an exhaustive list of genes that govern the host response to the gut microbiota. The functional studies of these candidates will immensely advance our understanding of the molecular basis of host-microbiota interaction. Furthermore, how are these host transcriptomic changes integrated into the known insulin and TOR signalling networks - as a response to the gut microbiota - to control systemic growth and metabolic homeostasis? Similarly, are these transcriptomic changes directly mediated by unknown bacterial factors, or through bacterial modification of the food substrate, or both? If both, what are the bacterial factors and how is the food modified? These are immediate questions that can be addressed with gnotobiotic models coupled to metabolomics and mutagenesis studies.

The gut microbiota impacts social behaviour

Throughout the long eukaryotic evolutionary history, many animal species abandoned the solitary life style for group living in highly developed social structures, in exchange for bodily protection, cooperative foraging and increased chances of mating and reproduction. As the long-time evolutionary partner of its eukaryotic host, it is not surprising that the symbiotic gut bacteria also evolved to control host individual and social behaviour, probably with the interest to maximize its transmission among the members of the society (*Montiel-Castro* et al., 2013). Through bidirectional signalling along the "microbiota-gut-vagus-brain axis", the activities of the gut microbiota can impact the activities of host neural circuitry and alter host foraging behaviour, stress and anxiety response and even the development of empathy (for

extensive reviews, see: *Cryan* and *Dinan*, 2012; *Montiel-Castro* et al., 2013; *Stilling* et al., 2014). Gnotobiotic flies have recently emerged to be a productive model to study social interactions. For example, fruit flies preferentially mate with partners fed on the same kind of diet, a phenomenon termed "positive assortive mating", which is readily lost in axenic flies. However, the gnotobiotic addition of *L. plantarum* restores such positive assortive mating, indicating that the gut microbiota may play a direct role in altering fly pheromone composition according to the host's dietary environment (*Sharon* et al., 2010). Besides mating preference, the presence of gut microbiota was also shown to determine how fruit flies are attracted by odours from different food substrates (*Venu* et al., 2014). In controlled learning experiments, Venu et al. presented the larvae subjects with three separate food choices: fresh laboratory food, food processed by axenic larvae, and food used by conventionally reared larvae. While the larvae and adult female subjects showed no preference between the fresh food and axenically processed food, they were strongly attracted to the food substrate where the CR larvae were raised. Furthermore, the same larvae subjects equally preferred food that has been used to raise larvae mono-associated with *Lactobacillus brevis* or *L. plantarum*. As an important control, the same larvae subject showed no preference to fly food containing only cultured *L. brevis*, indicating the interaction between *L. brevis* or *L. plantarum* with the fly larvae is imperative to generate the source for such social attraction. The nature of such source is unknown, but it can be a volatile compound produced by either the bacteria or the larvae when both are residing in the same niche (Figure 2). In the wild, fruit flies search of hospitable habitat with suitable food substrate for mating, egg laying and rearing larvae (*Durisko* and *Dukas*, 2013). The results from this study imply that the host interaction with the commensal *Lactobacillus* genus of the gut microbiota can manufacture compounds that serve as cues for the host's searching effort and decision-making. What are these compounds? Through what pathways and neurons do they act? What other aspects of fly behaviour do they affect? These are the questions that probably can also be answered with gnotobiotic studies.

CONCLUSION

In a recent essay, McFall-Ngai and colleagues commented that we humans are just "animals in a bacterial world" (*McFall-Ngai* et al., 2013). This pithy statement rightly illustrates the overwhelming number and the diversity of the microbes that we live with, yet we have only began to grasp how these seemingly humble dwellers can powerfully change our being throughout evolution. By enhancing the host's metabolic potential, the gut microbiota helps expand the host's ecological niche. By altering host behaviour, these bacteria probably also played their parts in shaping social hierarchies and caste systems in the animal kingdom. We still know very little about how the bacteria do it. However, by harnessing the power of the gnotobiotic flies, we have begun to systemically characterize how the gut microbiota potently elicits a myriad of host physiological responses and behavioural changes. Importantly, the gnotobiotic model, in combination with classic genetics and

large-scale next generation sequencing methods, grant us the unprecedented power of resolution to pinpoint the specific bacterial factors responsible a particular host phenotype. Only with such resolution, we can delve deeper into the mechanisms that govern host-microbiota interaction, and find answers to how these mechanisms evolved over time in different species. However, no matter how complex and unexpected these answers are, they never will deviate from the truth that Pasteur and Wollman prompted us to discover, that our genetic makeup is metagenomic, and our life story is indispensably, microbial.

LITERATURE

Bakula, M.: The persistence of a microbial flora during postembryogenesis of Drosophila melanogaster. J. Invertebr. Pathol. 14, 365-374 (1969).

Begg, M. and Sang, J.H.: A method for collecting and sterilizing large numbers of Drosophila eggs. Science 112, 11-12 (1950)..

Blum, J.E., Fischer, C.N., Miles, J., and Handelsman, J.: Frequent replenishment sustains the beneficial microbiome of Drosophila melanogaster. mBio 4, e00860-13 (2013).

Broderick, N.A. and Lemaitre, B.: Gut-associated microbes of Drosophila melanogaster. Gut Microbes 3, 307-321 (2012).

Broderick, N.A., Buchon, N., and Lemaitre, B.: Microbiota-induced changes in Drosophila melanogaster host gene expression and gut morphology. mBio 5, e01117-14 (2014).

Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S., and Lemaitre, B.: Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe 5, 200-211 (2009).

Buchon, N., Silverman, N., and Cherry, S.: Immunity in Drosophila melanogaster--from microbial recognition to whole organism physiology. Nat. Rev. Imunol. 14, 796-810 (2014).

Chandler, J.A., Lang, J.M., Bhatnagar, S., Eisen, J.A., and Kopp, A.: Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. 7, e1002272 (2011).

Chaston, J.M., Newell, P.D., and Douglas, A.E.: Metagenome-wide association of microbial determinants of host phenotype in Drosophila melanogaster. mBio 5, e01631-14 (2014).

Chouaia, B., Gaiarsa, S., Crotti, E., Comandatore, F., Degli Esposti, M., Ricci, I., Alma, A., Favia, G., Bandi, C., and Daffonchio, D.: Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts. Genome Biol. Evol. 6, 912-920 (2014).

Clemente, J.C., Ursell, L.K., Parfrey, L.W., and Knight, R.: The impact of the gut microbiota on human health: an integrative view. Cell 148, 1258-1270 (2012).

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., Negri, I., Scuppa, P., Rossi, P., Ballarini, P., Raddadi, N., Marzorati, M., Sacchi, L., Clementi, E., Genchi, M., Mandrioli, M., Bandi, C., Favia, G., Alma, A., and Daffonchio, D.: Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. Environ. Microbiol. 11, 3252-3264 (2009).

Cryan, J.F. and Dinan, T.G.: Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13, 701-712 (2012).

Danielsen, E.T., Moeller, M.E., and Rewitz, K.F.: Nutrient signaling and developmental timing of maturation. Curr. Top. Dev. Biol. 105, 37-67 (2013).

Douglas, A.E.: Symbiosis as a general principle in eukaryotic evolution. Cold Spring Harb. Perspect. Biol. 6, a016113 (2014).

Durisko, Z. and Dukas, R.: Attraction to and learning from social cues in fruitfly larvae. Proc. Biol. Sci. 280, 20131398 (2013).

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A.: Diversity of the human intestinal microbial flora. Science 308, 1635-1638 (2005).

Erkosar, B., Storelli, G., Defaye, A., and Leulier, F.: Host-intestinal microbiota mutualism: "learning on the fly". Cell Host Microbe 13, 8-14 (2013).

Erkosar, B. and Leulier, F.: Transient adult microbiota, gut homeostasis and longevity: novel insights from the Drosophila model. FEBS Lett. 588, 4250-4257 (2014).

Fond, G., Boukouaci, W., Chevalier, G., Regnault, A., Eberl, G., Hamdani, N., Dickerson, F., Macgregor, A., Boyer, L., Dargel, A., Oliveira, J., Tamouza, R., and Leboyer, M.: The "psychomicrobiotic": Targeting microbiota in major psychiatric disorders: A systematic review. Pathol. Biol. (Paris) 63, 35-42 (2014).

Fridmann-Sirkis, Y., Stern, S., Elgart, M., Galili, M., Zeisel, A., Shental, N., and Soen, Y.: Delayed development induced by toxicity to the host can be inherited by a bacterial-dependent, transgenerational effect. Front. Genet. 5, 27 (2014).

Gibson, M.K., Pesesky, M.W., and Dantas, G.: The Yin and Yang of bacterial resilience in the human gut microbiota. J. Mol. Biol. 426, 3866-3876 (2014).

Grenham, S., Clarke, G., Cryan, J.F., and Dinan, T.G.: Brain-gut-microbe communication in health and disease. Front. Physiol. 2, 94 (2011).

Gronke, S., Clarke, D.F., Broughton, S., Andrews, T.D., and Partridge, L.: Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genet. 6, e1000857 (2010).

Guo, L., Karpac, J., Tran, S.L., and Jasper, H.: PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell 156, 109-122 (2014).

Hamilton, P.T. and Perlman, S.J.: Host defense via symbiosis in Drosophila. PLoS Pathog. 9, e1003808 (2013).

Hietakangas, V. and Cohen, S.M.: Regulation of tissue growth through nutrient sensing. Annu. Rev. Genet. 43, 389-410 (2009).

Human Microbiome Project Consortium: Structure, function and diversity of the healthy human microbiome. Nature 486, 207-214 (2012).

Ikeya, T., Broughton, S., Alic, N., Grandison, R., and Partridge, L.: The endosymbiont Wolbachia increases insulin/IGF-like signalling in Drosophila. Proc. Biol. Sci. 276, 3799-3807 (2009).

Irrazabal, T., Belcheva, A., Girardin, S.E., Martin, A., and Philpott, D.J.: The multifaceted role of the intestinal microbiota in colon cancer. Mol. Cell. 54, 309-320 (2014).

Kaiko, G.E. and Stappenbeck, T.S.: Host-microbe interactions shaping the gastrointestinal environment. Trends Immunol. 35, 538-548 (2014).

Karlsson, F., Tremaroli, V., Nielsen, J., and Backhed, F.: Assessing the human gut microbiota in metabolic diseases. Diabetes 62, 3341-3349 (2013).

Lemaitre, B. and Miguel-Aliaga, I.: The digestive tract of Drosophila melanogaster. Annu. Rev. Genet. 47, 377-404 (2013).

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Loso, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J.G., Tautz, D., and Wernegreen, J.J.: Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. USA 110, 3229-3236 (2013).

Montiel-Castro, A.J., Gonzalez-Cervantes, R.M., Bravo-Ruiseco, G., and Pacheco-Lopez, G.: The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Front. Integr. Neurosci. 7, 70 (2013).

Natarajan, N. and Pluznick, J.L.: From microbe to man: the role of microbial short chain fatty acid metabolites in host cell biology.

Am. J. physiol. Cell Physiol. 307, C979-C985 (2014).

Newell, P.D. and Douglas, A.E.: Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. Appl. Environ. Microbiol. 80, 788-796 (2014).

O'Hara, A.M. and Shanahan, F.: The gut flora as a forgotten organ. EMBO Rep. *7*, 688-693 (2006).

Pasteur, L.: Observation relative à la note précédente de M. Duclaux. Compt. Rend. Acad. Sci. 100, 68 (1885).

Rastall, R.A.: Bacteria in the gut: friends and foes and how to alter the balance. J. Nutr. 134, 8 Suppl., 2022S-2026S (2004).

Reuter, G.: The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr. Issues Intest. Microbiol. 2, 43-53 (2001).

Ridley, E.V., Wong, A.C., Westmiller, S., and Douglas, A.E.: Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. PloS One 7, e36765 (2012).

Ridley, E.V., Wong, A.C., and Douglas, A.E.: Microbe-dependent and nonspecific effects of procedures to eliminate the resident microbiota from Drosophila melanogaster. Appl. Environ. Microbiol. 79, 3209-3214 (2013).

Ringo, J., Sharon, G., and Segal, D.: Bacteria-induced sexual isolation in Drosophila. Fly (Austin) 5, 310-315 (2011).

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I., and Rosenberg, E.: Commensal bacteria play a role in mating preference of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 107, 20051-20056 (2010).

Sharon, G., Garg, N., Debelius, J., Knight, R., Dorrestein, P.C., and Mazmanian, S.K.: Specialized metabolites from the microbiome in health and disease. Cell Metab. 20, 719-730 (2014).

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J.: Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334, 670-674 (2011).

Sommer, F. and Backhed, F.: The gut microbiota--masters of host development and physiology. Nat. Rev. Microbiol. 11, 227-238 (2013).

Stilling, R.M., Dinan, T.G., and Cryan, J.F.: Microbial genes, brain & behaviour - epigenetic regulation of the gut-brain axis. Genes Brain Behav. 13, 69-86 (2014).

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F.: Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab. 14, 403-414 (2011).

Tremaroli, V. and Backhed, F.: Functional interactions between the gut microbiota and host metabolism. Nature 489, 242-249 (2012).

Venu, I., Durisko, Z., Xu, J., and Dukas, R.: Social attraction mediated by fruit flies' microbiome. J. Exp. Biol. 217, 1346-1352 (2014).

Walter, J., Britton, R.A., and Roos, S.: Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. Proc. Natl. Acad. Sci. USA 108, Suppl. 1, 4645-4652 (2011).

Wollman , E.: Sur l' élevage des mouches stériles. Contribution à la connaissance du rôle des microbes dans les voies digestives. Ann. Inst. Pasteur (Paris) 25, 79-88 (1911).

Wong, A.C., Dobson, A.J., and Douglas, A.E.: Gut microbiota dictates the metabolic response of Drosophila to diet. J. Exp. Biol. 217, 1894-1901 (2014).

Wong, C.N., Ng, P., and Douglas, A.E.: Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. 13, 1889-1900 (2011).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 33-48 (2017).

GENERAL RULES FOR ESTABLISHMENT AND MAINTENANCE OF GASTROINTESTINAL ECOSYSTEMS

TORE MIDTVEDT

Department of Microbiology, Tumor and Cell Biology,
Karolinska Institute, Stockholm, Sweden

INTRODUCTION

The main title of this seminar ("The Magnificent Microbiome - Future Aspects") strongly indicates a holistic view on present and future aspects related to our magnificent microbiome. Therefore, I am allowing myself the liberty of taking a broad holistic view on the general rules for establishing and maintenance of the life-long interactions going on between a host organism and its microbiota, thus making truth to a general opinion:

- Man and his microbiota = a super-organism,
- Our gastrointestinal tract and its microbiota = a super-organ.

WHAT IS ECOLOGY?

The word comes from the Greece "*oicos"*, meaning "home". According to *Begon* et al. (2006) the word ecology was firstly used by Ernest Haeckel in 1869 to describe studies of interactions between organisms and their surroundings. Simplified (and with focus on functions), ecosystems can be defined as the co-operation going on between species, strains or individuals and their surroundings in a defined area. In even more popular words, ecology describes life as it is lived.

In medical sciences - contrary to ecological sciences - the focus has often been on the organism. Doctors and medical scientists have in the greatest detail studied sub-cellular particles, cells, tissues, organs and the organism itself. Ecology is in fact dealing with another level, starting with the individual organism, followed by the population (consisting of individuals of the same strain or species), the community (consisting of a certain number of strains or species) and the environment. At all these levels, a continuous flow of interactions takes place. The sum of species and interactions in a community present in a defined area is often characterized as an ecosystem. It should be kept in mind that an ecosystem is never in balance, but always in some sort of a balanced unbalance. The description given above opens up for studies at different levels

At the level of the organism, ecological studies are most often dealing with how an individual member is affected by, and how it affects, the environment. At this levels environment consists of all factors outside the organism irrespectively whether they are other organisms or species (biotic), physical or chemical (abiotic) factors.

At the level of the population, ecological studies are focused upon presence or absence of particular species, their abundance or rarity, and upon trends and fluctuation in their number. In fact, up to now, such studies have created the dominant part in medical ecology.

At the level of community, ecologi-

cal studies are focused upon the organization of various species within a defined area or compartment. Studies of biofilms may be taken as typical community ecological studies and they have become an important part of modern medicinal ecology.

In all these three approached, the focus is often mostly descriptive, i.e. which species are there, fluctuations in numbers, specific relationships in arrangements, etc., etc., leaving variations and interactions in functions as well as interactions with biotic and abiotic environmental factors relatively little commented upon. In 1992 Liekens tried to include in the definition of ecology "…the interactions between organisms and the transformation and flux of energy and matter" (*Liekens*, 1992). Although this extension in definition has not been accepted generally, it goes without saying that "the flux of energy and matters" are two important principles in establishment and maintenance in all ecosystems.

GENERAL THEORIES AND RULES FOR DEVELOPMENT OF ECOSYSTEMS

Irrespectively of whether looking upon development of ecosystem(s) from an evolutionary point of view (example: evolution of life on earth) or from an individual point of view (example: evolution of a "microbiota" in an individual at birth), the principles might be the same but the problems might be different. Per definition, the term "microbiota" includes all of the bacteria, viruses, fungi and protoctists that are present in a specific ecosystem. However, it has to be underlined that up to now, most studies on human/microbe ecosystems have been on the bacteria present at a particular site. In fact, at present we know much less about viruses, fungi and protoctists within human/microbe ecosystems.

In a very recent publication (*Pintor* et al., 2011) the authors used the so-called evolutionary game theory for studying and expressing the going-on in every ecosystem. In evolutionary game theory, the concept evolutionary stable strategy (ESS) is used to describe the set-up or sum of strategies used by existing members of an ecosystem to prevent the establishment of a newcomer. They used the so-called fitness-generating (G-function) approach to distinguish among three possible pathways: novel evolutionary strategy, empty niche, and recipient community non-evolutionary stable strategy. They define G-function as "the per capita growth rate and the evolutionary dynamics of a species possessing a particular strategy within a particular environment".

Applying their concept on an individual level at birth, the three following examples might be pertinent. A bacterial species capable to adhere to an epithelial cell has a novel strategy for a newcomer to be established in an ecosystem in which the "native" species do not possess this capability. Bacterial species capable of utilizing hydroxyl groups present in many host-derived compounds delivered to the intestinal tract, as bile acids, steroids, bilirubin etc., represent "specialists" capable of filling out niches not reached by the great majority in the intestinal microbiota, whereas strains – or species – multiplying a little bit faster than existing species represent a recipient community non-evolutionary stable strategy.

Without going into any evaluation of their mathematical modelling, it is easy to follow the authors when they

state that the ESS concept provides a new mechanistic hypothesis for when entrance of a newcomer results in long- or short-term increases in biodiversity and/or species replacement. A major objection is the ESS term itself: "evolutionary stable strategy". As mentioned above, an ecosystem is never in balance, but always in some sort of a balanced unbalance ("give me a solid point, and I shall move the Earth"), creating an initial uncertainty in mathematical evaluations.

TERMS OF EXCLUSIONS AND INCLUSIONS

A variable degree of resistance – and acceptance – to a newcomer seems to be a general feature in all ecosystems. In medical ecology, most attention has been focused upon mechanism(s) of value for exclusion of newcomers thought to be able causing diseases, whereas far little attention has been paid to mechanism(s) behind acceptance. In general terms, these two features or functions, i.e. exclusion or inclusion of newcomers in any ecosystem, can be defined as follows:

- *Colonization resistance (CR)* is a function to be found in all ecosystems, representing the sum of factors exhibiting a newcomer to be established.
- *Colonization conductance (CC)* is a function to be found in all ecosystems, representing the sum of factors allowing a newcomer to be established.

In the following these two functions will be focused upon from a medical point of view, with emphasis on the intestinal microbiota (IM) of mammals, including man.

In 1916, the German physician G. Nissle reported that a human bacterial gut sample was able to reduce growth of a pathogenic *Salmonella* strain *in vitro* (*Nissle*, 1916). He also reported that this antagonistic effect was changing according to the intestinal samples that he tested. He created an "antagonistic index" to ranking these samples, and selected one strain of *Escherichia coli* for further testing. In some following experiments he showed that this strain, later named *E. coli* Nissle, was able to cleanse otherwise healthy human typhoid carriers for their salmonella (i.e. biotherapy) and also made it difficult for new pathogens to be established. However, he never worked out or hypothesized about the mechanism(s) behind these antagonistic effects. The Nissle strain is still on the market in many countries, especially in Europe.

A new situation was created in 1929, when Alexander Fleming reported on production of an inhibitory substance, penicillin, made by a mould (*Fleming*, 1929). Since then, many bacteriocins and microbiocins, capable of interfering with the growth of other microbial species have been isolated and characterized. Some of them have been introduced in medical therapy as antibiotics, and it is well known, indeed, that many of these may cause serious alterations in IM.

In the 1970ties, different groups of researchers started to work more specifically to unravel the inhibitory effects of IM itself and several synonyms entered the scientific arena. *Greenberg* et al. (1970) reported on a *"natural resistance"* of IM in flies, capable of protecting them against salmonella; *Freter* and *Abrams* (1972) explored the mechanism(s) of the "control function" by which mouse IM antagonized the establishment of some invading micro-

organisms as *Shigella.* They emphasized that a co-operation between different microorganisms was necessary in their mouse model. Dirk van der Waaij and co-workers found that mouse IM provided a "colonization resistance" against several exogenous microorganisms and observed a weakening effect of some antibiotics on that function (*van der Waaij* et al., 1971).

Since then, many attempts have been made to find the strains or species in charge of CR. The aerobic as well as the anaerobic part of IM have been investigated and, so far, with very limited success. However, a weakening effect of many antibiotics upon CR is a well-established fact (*Barza* et al., 1987). In addition, a positive spin-off effect of these attempts is that we now have a better understanding of some of the complex cross-talks that continuously are going on between members in the microbiota at various places on a mammalian organism and host.

The term *colonization conductance* is new in ecology. In fact, a young scientist, Daniel Midtvedt, introduced the term to me when we discussed establishment of a microbial production of nitric oxide in fish, especially cod. The term reflects, in a positive way, the continuous balanced unbalance that always takes place in all ecosystems, driven by "the flux of energy and matter", thereby allowing a newcomer to find its place and be established.

However, irrespectively of using the CR or the CC concept for describing the possible fate of a newcomer into an existing ecosystem, a key problem has been to find suitable biomarker(s) for quantification. Some decades ago much attention was paid to faecal presence of a beta-aspartylglycine. Absence/presence of that dipeptide was assumed to reflect an adequate/reduced CR, respectively. However, further investigation showed that assumption to be too simplified. Realizing the complexity and dynamic alterations in most ecosystems, general biomarkers might be difficult to find.

All ecosystems are characterized by a continuing exchange of information between members within the system. In mammalian/microbial systems, information – or interactions – might be host/microbe-, microbe/microbe- or host/host-related. These interplays are governed by numerous factors as host genome and microbiome, epigenetic systems participating in gene expression and post-translated modifications of gene products, cell-to-cell signalling, either by direct contact (lectins, see later) or by extracellular signalling substances. As recently summarized (*Shenderov*, 2007) this equilibrium is often disturbed by exogenous factors as antibiotics, antiseptic agents, food additives, pesticides, industrial pollutants, other chemicals and so on. These factors may have a variable degree of influence upon individual species present in the ecosystem(s) under exposure. Thus, human/microbe related ecosystems are never in balance, but always in some sort of a balanced unbalance.

Signalling substances

In attempts to unmask these complex interactions, germ-free animals exposed to single microbial species have often been utilized, exemplified as follows (*Bry* et al., 1996). At an age of about 3 weeks, enterocytes from both new-born conventional and germ-free mice were found to express fucocylated glycoproteins on their luminal surfaces. However, at 3 months of age, the germ-free enterocytes had switched off this production. When 3-month-old germ-free mice were mono-associated with a fucose-utilizing strain of *Bacteroides thetaiotamicron*, their enterocytes rapidly switched on the production again whereas mono-association with an iso-

genic strain carrying a transposon insertion that disrupts its ability to use fucose as a carbon source did not cause any switch on. This rapid *cross-talk* was carried out by signalling substances that are not yet fully characterized. It has also been shown that the cross-talk is not dependent on living *B. thetaiotamicron* and that this strain can switch on production on several similar substances (*Freitas* et al., 2005).

Lectins

All types of cells, irrespectively of coming from the plant or animal kingdom, have so-called *lectins* on their surfaces. The word lectin comes from the Latin word "legere" which means "to select" and was introduced in biology by the British pathologist William Boyd in 1954. Basically, lectins are proteins/glycoproteins with at least one catalytic domain that exhibit – often reversible – binding to specific monosaccharides or oligosaccharides. They can be classified according to their overall structures into groups as chimerolectins, ficolectins, hololectines, merolectins, superlectins, etc., or grouped into different families (legume lectins, type II ribosome-inactivating proteins, mannose-binding lectins etc.) (*Lam* and *Ng*, 2011). Obviously, they play major roles in creating ecosystems, especially in what that has been called "behavioural ecology" (*Queller*, 2008). Currently, there is a considerable interest in rapid methods unmasking the "lectin profile" in various biological systems (*Chan* and *Ng*, 2010; *Lakhtin*, 2011; *Vandenborre* et al., 2011). It seems reasonable to assume that we are in the beginning of the beginning of a new era in which "we can successfully manipulate both the host and his microbiota through interfering in their cross talks, stability and epigenomic regulations of expression of genes" (*Shenderov*, 2011). It seems reasonable to assume that in the future this approach will give an increased preciseness and individualization in biomedical diagnosis and therapy (*Shenderov*, 2011; *Shukia* and *Tiwari*, 2011).

GENERAL DESCRIPTION OF HOST-RELATED MICROBIAL ECOSYSTEMS

A general feature in all multi-cellular organisms is that the outer surface and all openings going into the organism (as surface glands, respiratory and genito-urinary tract, etc.) are harbouring a microbiota. The inner parts of these openings are usually sterile. The alimentary tract is an exception since it is open in both ends and a microbiota can be present in all parts of the tract. In order to be established at any of the places mentioned, the environment must be able to satisfy the newcomer's physiochemical and nutritional requirements and the newcomer must on its side be able to withstand the various mechanical and hydro-dynamical microbe-removing systems present at certain sites (host cell desquamation, coughing, urinary flow, menstruation, motility, peristalsis, migration motor complexes, mucociliary movements, etc.).

Accepting that mammalian ecosystems most often are very complex, thereby making a mathematical evaluation presently close to impossible, it might be prudent taking a short glance at some simplified mathematical modelling. In general ecology, the combined Liebig-Shelford law has been found to be suitable (*Lystsov* and *Mur-*

zin, 2001). The Liebig paradigm of the minimum states that the total yield or biomass of any participant in an ecosystem is determined by the nutrient present in the lowest concentration in relation to the requirement of the participant whereas the Shelford paradigm of tolerance relates to the non-nutritional factors influencing upon the ecosystem. In any ecosystem, a particular participant will only survive if each of the physiochemical conditions operating there is within the tolerance range of that participant.

A hallmark for many human ecosystems is a large diversity. The following chapters will be focused upon some general factors influencing – and shaping – all human-related ecosystem in varying degrees.

TROPISM

Some microorganisms are only found in one or some few ecosystems, and this *tropism* can be tissue or compartment related. Basically, it represents the net sum of all environmental and host-related factors at the particular site.

Tissue tropism

The predilection of many microorganisms for a particular host site has been known for more than a century. This phenomenon is well established in clinical medical microbiology. A gonococcus is not to be expected as a cause of diarrhoea and a *Shigella* is not to be expected in a superficial wound on an arm, etc. As underlined by *Wilson* (2005), an understanding of such host-microbe interaction can be gained only by considering the anatomy and physiology of the site that is largely responsible for the unique environment existing there. Already Louis Pasteur stated: "the germ is nothing, it is the terrain in which it is found that is everything".

Compartment tropism

A general feature in all blind-ending surface openings in a multi-cellular organism is that the blind-end is usually sterile whereas ecosystems are established in the more surface-related parts of the openings (sweet glands, respiratory tract, urogenital tract etc.). Most often these ecosystems are very specific in their composition and therefore the term compartment tropism can be used.

A general feature is that the secretions through these openings contain the main elements needed for microbial growth, as carbon, nitrogen, minerals, etc. From a teleological point of view, it is reasonable to assume that the host invites microorganisms to be established for a proper breakdown – and sometimes also re-circulation – of the substances that are excreted.

FACTORS INFLUENCING UPON ESTABLISHMENT AND MAINTENANCE OF HUMAN ECOSYSTEMS

As mentioned above, all types of cells, irrespectively of coming from the plant or animal kingdom, have lectins on their surfaces. In the animal kingdom, cells are either of endodermic, mesodermic or ectodermic origin and cells belonging to the same line are often expressing similar lectins.

At all surfaces, two main types of epithelial surfaces can be distinguished

– dry epithelia (epidermis) covering the outer surface of the body and the moist epithelia which cover the eyes and all internal body surfaces that are in communication with the external environment (respiratory, gastrointestinal, urinary an genital tracts). Moist epithelia are often called mucosa as they are coated with a layer of glycoproteins known as mucins. The epithelial cells might be squamous, cuboid or columnar and they may form one or more layers. On their surface, epithelial cells as well as microorganisms have lectins, thus opening up for a cell-microbe tropism. In fact, in general as well as in medical ecology, lectins have important roles in shaping ecosystems (for references, see above).

A second factor of importance in tissue and compartment tropism is the rate of epithelial cell turnover. For some unknown reasons, this parameter is seldom brought up when human ecosystems are discussed. At any place, presence of a microbe might depend partly of its own multiplying capacity, partly on the longevity of the epithelial cells. The keratinous upper layer in skin might be there for weeks and even months, allowing slow-growing microorganisms, as fungi, to be established. In the small intestine, epithelial cell division is very rapid (*Banasaz* et al., 2000) and an enterocyte will live for just some few days (*Falk* et al., 1998). In all places of the small and large intestine there is a constant movement of cells from the mitotic compartment in the crypts to the surface where they are extruded, together with a flow of fluid and mucins.

A third factor of importance in compartment tropism is presence of host-produced defence peptides (HDPs), now often commonly called defensins. Such peptides are essential components in an ancient, non-specific innate defence system, representing a first line of host defence in insects, birds, reptils, mammals and plants. The first indications of their existence were brought forward decades ago (*Boman* et al., 1974). Since then more than a thousand of such peptides have been identified in plants, fungi, vertebrates and invertebrates (*Wilmes* et al., 2011). It has been postulated that defensins, especially those in plants and insects evolved from a single precursor (*Thevissen* et al., 2004). If so, they represent very ancient and basic eco-regulators. Vertebrate HDPs are often subdivided into 3 classes, mostly based on differences in spacing and pairing of six conserved cysteine residues (*Wilmes* et al., 2011). At first, the mode of action of HDPs was thought to result from electrostatic interaction between the positively charged HDPs and negatively charged microbial membranes. However, results of recent research strongly indicate that their activities can be much more targeted and that microbe-specific lipid receptors are involved in their killing profile. As many of the defensins, especially in mammalian gut, are produced as pro-defensins and have to be activated by proteases, I am allowing myself the liberty bringing forward a new theory *that some of these proteases might be of microbial origin giving the microbes an opportunity specifically to act upon host-derived weapons*. It has to be underlined this theory has never been tested.

A fourth factor of importance is host-related "local" motility. In all glands and in all tracts there is an inside-out flow of fluid; in the GI tract it goes oro-anally. The flow may partly be due to hydrodynamic factors, partly to host-related motility-enhancing principles. Here some of these motility-related factors will be viewed from an ecological point of view.

In the *respiratory tract* there exists a mucociliary escalator. In the posterior

two third of the nasal cavity, the nasopharynx and all the way down to the terminal bronchioles, ciliated cells are the most numerous cells in the epithelial lining. Each of them has approximately 200 cilia on their outer surface. The cilia beat in a sequential wave-like manner, and each cilium being in a slightly different stage in the beat cycle from its neighbour. The beat rate varies with the anatomical locations, but can be as high as 800 strokes/minute, and thereby propelling the mucus as fast as up to 20 mm/minute. Chemical alteration in mucus, as is the case in cystic fibrosis, may reduce transport of mucus and thereby induce alterations in the associated ecosystems.

In the *digestive tract* motility has several functions, as contributing to a physical breakdown of the food, mixing it with digestive secretions, propelling the mixture along the GI tract for absorption and final anal excretion. It has been known since long that intestinal transit time is longer in germ-free animals than in their conventional counterparts (*Abrams*, and *Bishop*, 1967). In a long series of comparative studies, Huseby and co-workers showed significant prolongation of migrating myo-electric complexes (MMCs) periods in germ-free compared with conventional rats (*Huseby* et al., 2001). Bacterial strains with an anaerobic fermentation profile, as Clostridia, Lactobacilli and Bifidobacteria, reduced the periods to nearly conventional values, whereas strains having an oxidative metabolic profile, as micrococci and *E. coli*, prolonged the periods. The biochemical mechanisms behind these effects were not investigated. However, whatever the mechanisms might be, it is reasonable to assume that alterations in MMC profiles in the small intestine will influence upon ecosystems within this part of the GI tract.

In the large intestine, the motility pattern is far more complex than in the small intestine. In many animal species, including man, there are segmentation contractions moving the content back and forth. Comparative studies in germ-free and conventional animals have shown that muscular sensitivity to biogenic amines is strongly influenced upon by presence of an intestinal microbiota (*Strandberg* et al., 1966).

Acute and chronic patho-physiological alterations in intestinal ecosystems, giving the host a lot of symptoms, are well known but a further evaluation is beyond the scope of this survey.

A fifth factor in compartmentalized tropism is the multiplication rate of the microbial strains present in that particular compartment. Surprisingly, there is very little information available about *in vivo* rate of division of most microbial species. When investigated, it seems to be slower than *in vitro.* Taking data from one of the few studies published it can be mentioned that in the GI tract of mice the generation time of *E. coli* was around one and a half hour and that multiplication took place in the ileo-coecal region (*Rang* et al., 1999). In another publication, the authors summarized their experience by stating that persistence of an *E. coli* population in the GI tract is promoted by species diversity and that "a mechanism for the persistence might be the presence of new *E. coli* niches created by keystone species in the most diverse flora" (*Rang* et al., 2001). It might be reasonable to assume that an "adjustment to alien genes" (*Johnson* and *Levin*, 2010) also may take place.

ANALYTICAL METHODS USED IN CHARACTERIZING MEDICAL MICROBIAL ECOLOGY

In evaluation of ecosystems involving both microbial and mammalian life, many *in* vitro as well as *in vivo* methods have been established. Microorganisms, occurring in pure culture or in complex mixtures, can be studied by various techniques as:

- Microscopy
- Culture dependent techniques
- Culture-interdependent techniques
- Functional studies

Microscopy

Light microscopy, either directly or as stained specimens, is the simplest and most direct approach for studying microorganism, either in pure culture or as parts of a microbial community. Over the years the analytic power has been enhanced in a number of ways. The use of vital stains can reveal the relative proportion of live and dead bacteria, fluorescent-labelled antibodies and labelled oligo-nucleotide probes can evaluate relationships between species present in an ecosystem and confocal laser scanning microscopy is a technique that enables us to study biofilms *in situ*, etc., etc. In the future, it seems reasonable to assume that capsule-cameras will enable us to study the microbial communities in the GI tract, especially in the small intestine, *in situ.*

Culture-dependent techniques

Most of our knowledge of the composition of the indigenous microbiota present in the ecosystems at various places on and in the human body came from qualitative and quantitative culture techniques. However, as already mentioned, for years the interest was focused upon isolation and identification of species assumed to be involved in diseases and far less attention was attributed to the problems of isolation and characterization of assumed normal microbiota. Realizing the complexity in most man-associated ecosystem, especially those in the alimentary tract, it seems reasonable to anticipate that we will never be able to cultivate all species present, even by utilization of a long variety of specific and selective media and culture conditions. As will be commented upon later, the oral cavity may be populated with 900 different species and our large colon with 1000-2000 species. World-wide, medical microbiological laboratories might be able to cultivate up to 20% of all species. It is time to be more humble. Campylobacter, one of the microbes often given rise to gastrointestinal problems, was recognized as a troublemaker less the 50 year ago, and *Helicibacter pylori*, present in the stomach of nearly 70% of all humans worldwide, was isolated and described in 1984.

Culture-independent technology

The rapid and huge developments of molecular technology that have taken place in the last few decades have circumvented many of the problems inherent in culture-based technology. So far, a key problem has been to create proper upsets of DNA or RNA probes supposed to cover the species in the ecosystems to be studied. New, rapid and steadily cheaper probe-independent technologies are now entering the market. In the future it is to be expected that we will be able to describe in great details all members in even that complicated ecosystem as the one in the large intestine. With similar improvements in bioinformatics as we have seen in microbial molecular technology we will have possibilities to solve many unsolved problems in human microbial ecology in health and diseases; i.e. will have answer to questions we presently are not able to ask.

So far, most of the culture-independent studies have been concentrated to describe the bacterial part of the microbiota within the alimentary tract. A future task will be to analyse the interactions of the other parts of the microbiota, i.e. bacteriophages, viruses, yeast, fungi and parasites, in the GI tract as well as in other human ecosystems. As stated by several, this area of research is likely to become increasingly important as more of the interkingdom signalling pathways are elucidated, and the importance of viral, parasite and fungal mutualism are recognized.

Functional techniques

Around a decade after Pasteur had made his famous state that "life is not possible without bacteria" two German scientists succeeded to keep a Caesarean derived guinea pig germ-free for some few weeks. Thus mammalian life was possible without bacteria. However, it took half a century until the second generation of germ-free animals was born at the University of Notre Dame, USA, in 1945. Then the possibility was created to clarify which structures and functions that are purely related to the host and which are influence upon by the microbiota, respectively. With a slight modification of terms first used by the French physiologist Claude Bernhard, the mammalian organism itself or the host's side of the ecosystem can be defined as *milieu interieur,* (MI) the microbial side as *milieu exterieur* (ME), and MI and ME together as *milieu total* (MT) (*Midtvedt*, 1999). Over the years, a long series of comparative studies in germ-free and conventional (i.e. organisms supposed to harbour a normal microbiota) mammals, birds, fish, reptiles and insects have established basal values for anatomical structure, and physiological, biochemical and immunological variables in MI and MT. When such structural and functional baselines are established, the normal function of the microbiota as well as alterations in the structure and/or functions under physiological and patho-physiological can be worked out. In such studies, two terms – Microbiota Associated Characteristics (MACs) and Germ-free Animal Characteristics (GACs) have been shown to be of considerable value (*Falk* et al., 1998, *Midtvedt*, 1999). A MAC is defined as the recording of any anatomical structure, physiological, biochemical or immunological function that has been influenced upon by the microbiota. When microorganisms influencing the variable under study are absent, as in a germ-free individual, new-borns or sometimes in adults (influenced upon by antibiotics act), values recorded are defined as GACs. Consequently, the sum of GACs found in a germ-free individual describes MI, and similarly, a sum of MACs describes MT. A simple equation MT minus MI gives ME: "what have the microbes done?".

To summarize, the MAC/GAC concept opened up a functional way of metabolic profiling, and can by definition be extended to all human-related materials for measurements of differences (serum, plasma, urine etc.). It creates the platform for metabolic profiling studies, most often carried out by mass spectrometry and NMR spectroscopic platforms. We are now under way in characterizing functional alterations in a wide variety of diseases as well as to establish biomarker screening procedures of aetiological, therapeutically and prognostic value (*Clayton* et al., 2006; *Teague* et al., 2007; *Holmes* et al., 2008; *Holmes* et al., 2011; *O'Sullivan* et al., 2011). Even more, utilization of modern technology allows us to follow subtle changes host-

microbe ecology thereby open up for a personalized therapy (*Nicholson* et al., 2011).

However, in spite of technological improvements in host-microbe related metagenomics and metabolomics we must admit that we still have a long way to go. Areas in which further research is of major ecological interest are being mentioned in the following chapters.

RESEARCH ON LABORATORY ANIMALS

At present, laboratory animals, especially mice and rats, are more and more used for unravelling the complicated cross-talks that continuously go on between mammalian hosts and their microbiota. Taking a broad overview on results, it is easy to find conflicting results, leading to uncertainties in interpretations. One reason for discrepancies might be variations in the ecosystems of the host.

In the 1950ties and 1960ties, comparative studies in conventional and germ-free animals clearly showed that the host's microbiota was responsible for the difference between those two groups. In a long series of experiments, carried out many places, bacteria strains or species capable of switching a host-related parameter from GAC to MAC status were described. As a consequence, a "dream" of a "minimum bacterial flora" was born. In the mid-1960ties, R.W. Schaedler selected 8 bacterial strains from "standard" (pre-SPF specific pathogen free) mice and claimed that this bacterial cocktail should protect laboratory animals, including germ-free, against infectious agents (*Schaedler* et al., 1965). Some years later, this cocktail was redesigned and given the name Altered Schaedlers flora (ASF) (*Orcutt* et al., 1985). It is worth mentioning that neither the original nor the ASF were designed for establishing any functional changes in ex-germ-free animals. The selection principle was "free of pathogenic microorganisms" and the major effect was protection from pathogens. Over the years breeders of laboratory animals, especially in the Unites State, used this ASF to inoculate Caesarean derived offspring of rodents and thereby fulfilling criteria of SPF status (specific pathogen free) established by the American Association of Laboratory Animal Science (AALAS) (https://www.aalas.org/), the Federation of European Laboratory Animal Science Associations (FELASA) (http://www.felasa.eu/) and other authorities. In the laboratories of the customers, these SPF animals can be kept for generations, most often under strict barrier conditions. Routine cultivation controls that may take place are always aimed for documentation of "free of pathogens". Up to now, a "positive list" a list covering which microbes that should be there, has never been published by any veterinary association or any regulatory authority.

Now it has clearly been shown that laboratory animals fulfilling the SPF status differ considerable from each other and from conventional individuals of the same species – in microbiological (*Wilson* et al., 2006), immunological (*Boysen* et al., 2011), and functional status (*Norin* and *Midtvedt*, 2010). Even when raised under barrier conditions, within the same breading facility the IM of the animals may vary considerably (*Hufeldt* et al., 2010a). Least variations were found in strictly controlled, family related offspring (*Hufeldt* et al., 2010b).

The bottom line of all these new results is that time has come to re-evalu-

ate present production of laboratory animals. SPF rodents reared under strict barrier conditions may represent "in-betweeners" when compared to germ-free and conventional rodents. The complexity in the composition of a host's indigenous microbiota and the many cross-talks that continuously are going on between the host and his microbiota demand a more precise definition of the latter. Otherwise, interpretations of results are difficult and may be misleading. A worst-case scenario is that they are valid just for that group of animals coming from that breeder. As underlined "time might have come for AALAS an FELASA to take a closer look into their SPF and ASF concepts" (*Norin* and *Midtvedt*, 2010). An adequate indigenous microbiota is far more than freedom from some pathogens.

SPECIFIC COMMENTS TO RULES FOR ESTABLISHMENT AND MAINTENANCE OF GASTROINTESTINAL ECOSYSTEMS

Intra-uterine epigenetic programming and/or presence of a placental microbiome

Very recently and based upon presence of microbial genetic material, many derived from bacterial species living in the oral area, it has been claimed that – during healthy pregnancies – there might exists living microbes in the uterine wall and/or placenta. However, presence of a live utero/placental microbiota is far from established. It should be kept in mind that translocation of bacterial products and even living bacteria from the oral cavity is a physiological process, and the filtration function of placenta is very well established, indeed. Additionally, presence of a placental microbiome would make it almost impossible to establish germ-free animals and this is certainly not the case.

Present view on the human microbiome, too far too fast?

During the last few years numerous reports, utilizing modern molecular technology, have appeared describing human microbiomes, most often related to IM. Although a general consensus "about the phylum level composition is emerging (*Eckburg* et al., 2005; *Lay* et al., 2005, *Zoetendal* et al., 2008) the variation in species composition (*Eckburg* et al., 2005) and gene pools (*Qin* et al., 2010) within the human population is less clear. This was the background for a recent study in with 52 scientists combined their data from 22 newly sequenced faecal metagenomes of individuals from four European countries with previous published data sets from the Unites States and Japan (*Arumugam* et al., 2011). Their results indicate the presence of three distinct clusters or enterotypes. It goes without saying that the publication caused great interest. However, it should be underlined what the authors stated: "as our current data do not reveal which environmental and even genetic factors that are causing the clustering, and as faecal samples are not representative for the entire intestine, we anticipate that the enterotypes introduced here will be refined with deeper and broader analysis of individual's microbiomes". It seems appropriate to state that the present 3 enterotypes represent a way of thinking more than a final conclusion. Hopefully, a similar approach will be of value when investigating the ecosystems present at other places of the mammalian body.

SHORT SUMMARY OF DEVELOPMENT OF INTESTINAL MICROBIOTA THROUGH INFANCY

"Man is born germ free". This axiom is still generally valid. However, from the first second of our life, irrespectively of whether we are born naturally or by Caesarean section, microbes will start entering the GI tract and continue to do so throughout infancy. It is generally assumed, but never satisfactorily shown, that during that period of time more than 2000 microbial species have been in the GI tract for a shorter or longer period of time. In a broad concept, three major factors will influence upon their establishment and also upon possible consequences for the host:

1. Windows for establishment.
2. Succession in establishment.
3. Long term effect(s) of establishment.

Att. 1: It has been known for more than a century that the microbiota in the GI tract of vaginally delivered new-borns is dominated by aerobic species, simply because the oxygen tension and reduction/oxidation potential (Rh) is high. It has also been shown decades ago that some functionally active microbes have to be established within weeks or months; if this happens later their function(s) might never be expressed.

Att. 2: It is an experience in gnotobiotic research that it is close to impossible to establish some very strict anaerobes as mono-contaminants. This has also observed in new-born babies. The first arriving aerobes will reduce oxygen tension and Rh, thereby creating improved conditions for more anaerobic species, as bifidobacteria and others, to be more permanently established.

Att. 3: When established, it is well known that under physiological conditions, all major groups (clostridia, bacteroides, para-bacteroides etc.) will be present in all healthy humans and so will also their major functions (hydrolysis of carbohydrates, production of short chain fatty acids and peptides, etc. However, it is also well known that several types of influences, as starvation, infections, antibiotics, etc., may cause major alterations in presence as well as in functions. Restoring a symbiotic IM in infants is, however, out of the scope for this short overview.

CONCLUDING REMARKS

As mentioned above we have now suitable methods for describing human ecosystems in great details, both regarding presence of microbes as well as presence of functions. Sometimes we may have a feeling that we have too many data to handle. It has been said that we have answers to questions we are not able to ask. The need of development in biostatistics is obvious. Additionally, utilization of well-established ecological theories and “laws”, as mentioned above, might also be a suitable way to go in order to avoid confounders and wrong conclusions. However, in spite of all present limitations, ***ecology is in the mid-stream of modern medicine.***

LITERATURE

Abrams, G.D. and Bishop, J.E.: Effect of the normal microbial flora on gastrointestinal motility. Proc. Soc. Exp. Biol. Med. 126, 301-304 (1967).

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

Banasaz, M., Aam, M., Morin, E., and Midtvedt, T.: Gender, age and microbial status influence upon intestinal cell kinetics in a compartmentalized manner. Microb. Ecol. Health Dis. 12, 208-218 (2000).

Barza, M., Guilrano, M., Jacobus, N.V., and Gorbach, S.L.: Effect of broad-spectrum parenteral antibiotics on “colonization resistance” of intestinal microflora in humans. Antimicrob. Agents Chemother. 31, 723-727 (1987).

Begon, M., Townsend, C.R., and Harper, J.L.: Ecology. From individuals to ecosystems. Fourth edition. Blackwell Publishing Ltd., Oxford, UK (2006).

Boman, H.G., Nilsson-Faye, I., Paul, K., and Rasmuson, T. Jr.: Insect immunity: Characteristics of an inducible cell-free antibacterial reaction in hemolymph of Samia Cynthia pupae. Infect. Immun. 10, 136-145 (1974).

Boysen, P., Eide, D.M., and Storset, A.K.: Natural killer cells in free-living Mus musculus have a primed phenotype. Mol. Ecol. 20, 5103-5110 (2011).

Bry, L., Falk, P.G., Midtvedt, T., and Gordon, J.I.: A model of host-microbial interactions in an open mammalian ecosystem. Science. 273, 1380-1383 (1996).

Chan, K. and Ng, T.B.: Lectin glycoarrays technologies for nanoscale biomedical detection. Prot. Pept. Lett. 17, 1417-1426 (2010).

Clayton, T.A., Lindon, J.C., Clorec, O., Antti, H., Charuel, C., Hanton, G., Provst, J.P., Le Net, J.L., Baker, D., Walley, R.J., Everet, J.R., and Nicholson, J.K.: Pharmacometabonomic phenotyping and personalized drug treatment. Nature 440, 1073-1077 (2006).

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Fill, S.R., Nelson, K.E., and Relman, D.A.: Diversity of the human intestinal microbiota. Science 308, 1635-1638 (2005).

Falk, P.G., Hooper, L.V., Midtvedt, T., and Gordon, J.I.: Creating and maintaining the gastrointestinal ecosystem; what we know and need to know from gnotobiology. Microbiol. Mol. Biol. Rev. 62, 1157-1170 (1998).

Fleming, A.: On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. Br. J. Exp. Pathol. 10, 226-236 (1929).

Freitas, M., Axelsson, L.G., Cayuela, C., Midtvedt, T., and Trugnan, G.: Indigenous microbes and their soluble factors differentially modulate intestinal glycosylation steps in vivo. Use of a “lectin assay” to survey in vivo glycosylation changes. Histochem. Cell Biol. 124, 423-433 (2005).

Freter, R. and Abrams, G.D.: Function of various intestinal bacteria in converting germfree mice to the normal state. Infect. Immun. 6, 119-126 (1972).

Greenberg, B., Kowalski, J.A., and Klowden, M.J.: Factors affecting the transmission of

salmonella by flies: natural resistance to colonization and bacterial interference. Infect. Immun. 2, 800-809 (1970).

Holmes, E., Loo, R.L., Stamler, J., Bictash, M., Yap, I.K., Chan, O., Ebbels, T., De Iorio, M., Brown, I.J., Veselkov, K.A., Daviglus, M.L., Kesteloot, H., Ueshima, H., Zhao, L., Nicholson, J.K., and Elliot, P.: Human metabolic phenotype diversity and its association with diet and blood pressure. Nature 453, 396-400 (2008).

Holmes, E., Li, J.V., Athanasiou, T., Ashrafian, H., and Nicholson, J.K.: Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. Trends Microbiol. 19, 349-359 (2011).

Hufeldt, M.R., Nielsen, D.S., Vogensen, F.K., Midtvedt, T., and Kornerup, A.K.: Variation of the gut microbiota in mice is related to both genetic and environmental factors. Comp. Med. 60, 336-342 (2010a).

Hufeldt, M.R., Nielsen, D.S., Vohensen, F.K., Midtvedt, T., and Hansen, A.K.: Family relationship of female breeders reduces the systematic inter-individual variation in the gut microbiota of inbred laboratory mice. Lab. Anim. 40, 283-289 (2010b).

Husebye, E., Hellström, P.M., Sundler, F., Chen, J., and Midtvedt, T.: Influence of microbial species on small intestine myoelectric activity and transit in germ-free rats. Am. J. Physiol. Gastrointest. Liver Physiol. 280, 368-380 (2001).

Johnson, P.J. and Levin, B.R.: Adjusting to alien genes. Mol. Microbiol. 75, 1061-1063 (2010).

Lam, S.K. and Ng, T.B.: Lectins: production and practical applications. Appl. Microbiol. Biothechnol. 89, 45-55 (2011).

Lakhtin, M., Lakhtin, V., Alvoskhin, V., and Afanasvev, S.: Lectins of beneficial microbes: system organization, functioning and functional superfamily. Benef. Microbes. 2, 155-165 (2011).

Lay, C., Rigottier-Gois, L., Holmstrom, K., Rajilic, M., Vaugham, E.E., de Vos, W.M., Collins, M.D., Thiel, R., Namsolleck, P., and Blaut, M.: Colonic microbiota signatures across five European countries. Appl. Environ. Microbiol. 71, 4153-4155 (2005).

Liekens, G.E.: The ecosystem approach: its use and abuse. Excellence in Ecology: Book 3. International Ecology Institute, Oldendorf-Luhe, Germany (1992).

Lystsov, V.N. and Murzin, N.V.: A strategy for the assessment of continuous and pulsed contamination in river and estuary ecosystems in the Arctic. Chemosphere 42, 73-78 (2001).

Midtvedt, T.: Microbial Functional Activities. In: Probiotics, other nutritional factors and intestinal microflora (Eds.: Hanson, L.A. and Yolken, R.H.). 42nd. Nestlé Nutrition Workshop, Lippincott-Raven Publishers, Philadelphia, USA, 10-11 (1999).

Nicholson, J.K., Wilson, I.D., and Lindon, J.C.: Pharmaco-metabonomics as an effector for personalized medicine. Pharmacogenomics 12, 103-111 (2011).

Nissle, G.: Über die Grundlagen einer neuen unsachlichen Bekämpfung der pathologischen Darmflora. Dtsch. Med. Wochenschr. 42,1181-1184 (1916).

Norin, E. and Midtvedt, T.: Intestinal microflora functions in laboratory mice claimed to harbor a "normal" intestinal microflora. Is the SPF concept running out of date? Anaerobe 16, 311-313 (2010).

Orcutt, R.O., Gianni, F.J., and Judge, R.J.: Development of an "altered Schadler Flora" for NCU gnotobiotic rodents. Microecol. Ther. 17, 59-62 (1985).

O'Sullivan, A., Gibney, M.J., and Brennan, L.: Dietary intake patterns are reflected in metabolic profiles: potential roles in dietary assessment studies. Am. J. Clin. Nutr. 93, 314-321 (2011).

Pintor, L.M., Brown, J.S., and Vincent, T.L.: Evolutionary game theory as a framework for studying biological invasions. Am. Nat. 177, 410-423 (2011).

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg,

A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J.; MetaHIT Consortium, Bork, P., Ehrlich, S.D., and Wang, J.: A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 50-65 (2010).

Queller, D.C.: Behavioral ecology: the social side of wild yeasts. Nature 456, 589-590 (2008).

Rang, C.U., Licht, T.R., Midtvedt, T., Conway, P.L., Chao, L., Kroghejt, K.A., Cohen, P.S., and Molin, S.: Estimation of growth rates of Escherichia coli BJ4 in streptomycin-treated and previously germfree mice my in situ rRHA hybridization. Clin. Diagn. Lab. Immunol. 6, 434-436 (1999).

Rang, R.U., Midtvedt, T., Molin, S., and Chao, L.: Chemostat modeling of Escherichia coli persistence in conventionalized mono-associated and streptomycin-treated mice. Can. J. Microbiol. 47, 86-90 (2001).

Schaedler, R.W., Dubos, R., and Costello, R.: Association of germfree mice with bacteria isolated from normal mice. J. Exp. Med. 122, 77-82 (1965).

Shenderov, B.A.: Modern conditions and prospective host microecological investigations. Microb. Ecol. Health Dis. 19, 145-149 (2007).

Shenderov, B.A.: Probiotic (symbiotic) bacterial languages. Anaerobe 1-6 (2011)

Shukia, R.K. and Tiwari, A.: Carbohydrate molecules: an expanding horizon in drug delivery and biomedicine. Crit. Rev. Ther. Drug Carrier Syst. 28, 255-292 (2011).

Strandberg, K., Sedvall, G., Midtvedt, T., and Gustafsson, B.: Effect of some biologically active amines on the cecum wall of germ-free rats. Proc. Soc. Exp. Biol. Med. 121, 699-702 (1966).

Teague, C.R., Dhabhar, F.S., Barton, R.H., Bechwith-Hall, B., Powell, J., Cobain, M., Singer, B., McEven, B.S., Lindon, C., Nicholson, J.K., and Holmes, E.: Metabonomic studies on the physiological effects of acute and chronic stress in Sprague-Dawley rats. J. Proteome Res. 6, 2080-2093 (2007).

Thevissen, K., Warneck, D.C., Francois, I.E., Leipelt, M., Heinz, E., Ott, C., Zähringer, U., Thomma, P.B., Ferkel, K.K., and Cammue, B.P.: Defensins from insects and plants interact with fungal glucosylceramides. J. Biol. Chem. 279, 3900-3905 (2004).

van der Waaij, D., Berghuis-de Vries, J.M., and Lekkerkerk-van der Wees, J.E.C.: Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg. (Lond.) 69, 405-411 (1971).

Vandenborre, G., Smagghe, G., and Van Damme, E.J.: Plant lectins as defense proteins against phytophagous insects. Phytochemistry 72, 1538-1550 (2011).

Wilmes, M., Cammue, B.P.A., Sahl, H.G., and Thevissen, K.: Antibacterial activities of host defense peptides: more to it than bilayer perturbation. Nat. Prod. Rep. 28, 1350-1358 (2011).

Wilson, K.H., Brown, R.S., Anderson, G.L., Tsang, J., and Sartor, B.: Comparison of fecal biota from specific pathogen free and feral nice. Anaerobe 12, 249-253 (2006).

Wilson, M.: Microbial inhabitants of humans. Their ecology and roles in health and disease. Cambridge University Press, Cambridge (2005).

Zoetendal, E.G., Rajilic-Stojanovic, M., and de Vos, W.M.: High-throughput diversity and functional analysis of the gastrointerstinal tract microbiota. Gut 57, 1605-1615 (2008).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 49-60 (2017).

EARLY COLONIZATION IN CAESAREAN CHILDREN

MERETE EGGESBØ[1], SIDDHARTHA MANDAL[2], SHYAMAL PEDDADA[3], and TORE MIDTVEDT[4]

[1]Norwegian Institute of Public Health, Nydalen, Norway;
[2]Public Health Foundation of India, New Delhi, India;
[3]National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA;
[4]Karolinska Institute, Stockholm, Sweden

GUT MICROBIOTA

Many diseases are increasing among children and young adults in the western world and changes in definition and increased detection rate cannot explain the increases in full. Environmental factors must be behind the increase as changes in genes evolve very slowly. Changes in human gut microbiota may be one of several environmental factors in part responsible.

A complex society of microbes exists in the human gut, and the collective genome of this microbiota greatly exceeds the number of genes in the human genome (*Backhed* et al., 2005) and we have come to understand its crucial role in host biology and human health. It may be considered as a separate organ with complex interplays with the immune system and the brain in particular.

The composition and functionality of gut microbiota in early life is of special interest due to several reasons. Firstly as a determinant for the subsequent adult-like microbiota, since the colonization of the gut is a dynamic process in which the selection of microbes will in part depend on the processes that have already taken place (*Midtvedt* et al., 1988; *Midtvedt* and *Midtvedt*, 1992; *Hooper* et al., 1999; *Thompson* et al., 2008; *Eggesbø* et al., 2011). In a study on 85 Norwegian new-borns multiple associations were observed between the presence of certain microbes or microbial groups at four days and the concentrations of microbes at four months, evaluated by the signalling by specific probes developed for that study (*Eggesbø* et al., 2011). Interestingly, the 23 microbes and/or microbial groups studied had very varying effect on the future colonization process, as evaluated by their associations to gut microbiota at four months (*Eggesbø* et al., 2011). Only rarely were the associations between the same microbes or microbial groups, with one exception: infants with initially high concentrations of *Bifidobacterium* were still characterized by higher concentrations of *Bifidobacterium* at four months of age. Also interesting was the finding that four days after birth the probe labelled *Lachnospiracea incertae sedis,* which detected species belonging to *Ruminococcus,* showed the highest number of associations with later microbiota (at four months).

Secondly, the composition of infant microbiota is important in its own right due to the presence of developmental windows in young age. Experimental animal studies in germ-free environments have demonstrated time-dependent windows that rely on microbial stimuli from the gut, with persistent dysfunction when microbial exposure is delayed beyond a certain age (*Desbonnet* et al., 2008; *Forsythe* et al.,

2010; *Neufeld* et al., 2011). Reducing microbial load in early life with antibiotics also induces lasting phenotypic differences in mice (*Cox* et al., 2014). However, very few studies in newborns yet exist. One study from our group aimed at identifying critical windows for exposure, by developing a novel method for longitudinal data (*White* et al., 2013).

ALTERATIONS IN INTESTINAL MICROBIOTA IN MODERN TIMES

There is increasing evidence to support that the composition of early microbial colonization communities have indeed changed during the last 50-100 years in western societies. The flora reported in western babies at the beginning of the 19th century, had amongst others a high count of *Bifidobacteria* and *E. coli* (*Escherich*, 1885; *Gareau* et al., 1959; *Nelson* and *Mata*, 1970; *Simhon* et al., 1982). While *E. coli* previously was found in all children by day 3 after birth, and a high turnover is still observed in infants from developing countries (*Gareau* et al., 1959; *Mata* et al., 1972; *Adlerberth* et al., 1998), it can no longer be detected in an increasing proportion of Swedish children (*Kühn* et al., 1986; *Adlerberth* et al., 1991; *Nowrouzian* et al., 2003; *Adlerberth* et al., 2006). In contrast, a variety of hospital acquired organisms, in particular *Staphylococcus sp.* and *Streptococcus sp*. seems to have partially replaced these organisms in western babies (*Lindberg* et al., 2000). *Staphylococcus sp.* were seldom encountered in Western infants up to the 1970s (*McAllister* et al., 1974; *Bullen* et al., 1976; *Stark* and *Lee*; 1982). In the 1980s, *S. epidermidis* was isolated from 30 to 70% of one-week-old Swedish infants (Lundequist et al., 1985; Bennet et al., 1991).

Streptococcus sp. colonization has been associated with lack of colonization by *Bifidobacteria* genera (*Eggesbø* et al., 2011). In contrast, the presence of Enterobacteriacea 1, and *Bacteroides fragilis* after birth was significantly associated with a reduction in opportunistic microbes at four months, specifically *Staphylococcus sp.*, indicating that the early presence of specific microbes may be important for development towards a more natural gut microbiota. If they are lacking, an initial colonization by opportunistic bacteria will occur and may also be maintained.

One of the limitations of old studies is that they had to rely on culture dependent methods, which limited the detection of anaerobic species in particular, and our knowledge of the natural composition of gut microbiota a century ago. Recent studies, on the other hand, do characterize gut microbiota by means of culture independent methods, however commonly fail to take into account factors that may greatly alter the composition of gut microbiota. Therefore the data are not representative of a normal gut microbiota in infants. One study from our group (*Eggesbø* et al., 2011) restricted the study population to:

- babies delivered vaginally by term;
- exclusively breastfed for at least one month;
- partially breastfed up to four months;
- no intensive care unit treatment;
- mothers who have not used antibiotics the month preceding delivery nor while breastfeeding (e.g. no antibiotic during the four months after birth) to limit microbiota disrupting factors to the highest degree possible.

This study, based on the NoMIC cohort (*Eggesbø* et al., 2011), used 23 specific

probes and confirmed that, even in infants with minimal medical interventions, the most common microbe colonizing the new born gut is *Staphylococcus sp*. This indicates that the changes in gut microbiota in the last 40 years may have occurred in the whole population. Exposures which affect gut microbiota, such as conservatives in food, traces of antibiotics in food and an ever-increasing sterile milieu, are ubiquitously present and may be hard to avoid in the western world.

In contrast, *Bifidobacteria* genera were common as expected and dominated in these breastfed infants at four months of age (*Eggesbø* et al., 2011). Moreover, the detection rate of *E. coli* was higher in this study (70% at day four) than among unselected populations of Scandinavian children, and increasing towards four months.

Interestingly, lack of *E. coli* was associated with rapid growth, a risk factor for later obesity, using the same 23 probes (*White* et al., 2013). This study reported on a novel method for identifying exposure windows. The continued presence or absence of *E. coli*, at 3 age points during the first month of life, was identified as being associated with infant growth pattern. In contrast presence or absence of *E. coli* later on was not associated with infant growth, indicating a critical window for exposure. This finding is supported by more recent studies indicating that *E. coli* plays a key role in regulating food intake (*Breton* et al., 2016) However, *E. coli* may serve as a marker for a less disrupted early gut microbiota.

CAESAREAN SECTION

Many factors tied to our modern lifestyle, such as hygienic measures, hospital delivery, use of antibiotics in the perinatal period and use of neonatal intensive care units, likely play a role in the shift observed in infant gut microbiota and neonatal intensive care. However, the single most important factor may be the increase in caesarean section (C-section) deliveries.

A sharp increase in C-section has occurred in most western countries. In the US a ten-fold increase in C-section rates was reported in the period from 1937 to 2005 and 30% are now delivered by a C-section (*Ecker* and *Frigoletto*, 2007). In Norway, there has been a seven-fold increase in C-section rates between the 1970s and 2001 (from 2 to 15%) (*Häger* et al., 2006). The reasons for this increase are complex and involve changes in recommendations, for instance for breech deliveries, as well as increase in the prevalence of high-risk pregnancies, such as obesity and multiple gestations. However, also changes in willingness to accept pain, is changing and many C-section are unnecessary from a strict medical point of view (*Ecker* and *Frigoletto*, 2007).

The increase in C-section rates has obvious medical consequences. Focus so far has mainly been on the immediate operative risks of a C-section as well as on the increased risk of adverse outcomes in subsequent pregnancies (*Duncan* and *Doyle*, 1937; *Liu* et al., 2007). However, C-section may have long-term consequences of a more subtle nature, tied to differences in the endocrine milieu during delivery, or to lack of input of microbes of maternal origin and these factors have so far been less acknowledged (*Steer* and *Modi*, 2009; *Hyde* et al., 2010).

There is increasing support for multiple long-term effects in C-section delivered children (*Cardwell* et al.,

2008; *Koplin* et al., 2008; *Thavagnanam* et al., 2008; *Amiri* et al., 2012; *Keag* et al., 2014). A recent systematic review concluded that children delivered by C-section have an increased risk of asthma, wheeze and obesity during childhood (*Keag* et al., 2014). Many papers, a meta-analysis and a review, support an association between C-section and food allergy (*Bager* et al., 2008; *Koplin* et al., 2008) where the finding of a stronger effect size among the infants with family risk supports a causal association (*Eggesbø* et al., 2003, 2005). Another meta-analysis finds support for an association between C-section and diabetes Type 1 (*Cardwell* et al., 2008). Associations have also been reported between C-section and childhood leukaemia (*Kaye* et al., 1991; *Cnattingius* et al., 1995), ADHD (*Amiri* et al., 2012), while a meta-analysis found no support for an association between testicular cancer and C-section (*Cook* et al., 2009). The underlying reason for the observed associations is not yet fully understood, and because C-section deliveries are associated with multiple pathological conditions in mother and child, the causes may be multifactorial. Studies indicate that early gut microbiota plays a key role in the regulation of the immune system and that babies delivered by C-section have less robust regulatory T-cell suppressive functions (*Ly* et al., 2006). Regulatory T-cells play a key function in the regulation of the immune system, and less robust regulatory T-cell function could result in immune-related diseases such as allergies or autoimmune diseases. Also, exposure to microbial components during birth has been shown to play a role for developing gut-epithelial tolerance to microbes, thus facilitating the subsequent colonization process (*Lotz* et al., 2006). Yet, there are other birth related processes, such as the natural peak in stress hormones and the compression of the brain in the birth canal, which are missed in a C-section, which also may play a role for the differential fate of C-section children. The hypothesis currently probably gaining most support is that it may be due to long-term effects of an altered early gut microbiota (GM).

THE EFFECT OF CAESAREAN SECTION ON GUT MICROBIOTA

Mammals have co-evolved with the microbial world throughout evolution. The foetus is to some degree exposed to maternal microbes already in the womb (*Aagaard* et al., 2014). Then a natural birth ensures massive transfer of commensals microbes from the mother to the child.

In C-section, exposure to bacteria in the maternal birth canal is bypassed, resulting in a delayed and highly disrupted infant GM, for instance resembling human skin rather than the mother's vaginal microbiota (*Dominguez-Bello* et al., 2010). This has been documented in a number of studies going back a century, using culture dependent methods as well as the more recent culture independent methods based on microbial DNA. They show that babies delivered by a caesarean section have a delayed and different colonization, with lower colonization rates of *Bifidobacterium* and *Bacteroides*, while rates of Clostridia and *Staphylococcus* are higher among caesarean delivered children (*Bennet* and *Nord*, 1987; *Neut* et al., 1987; *Grönlund* et al., 1999a, 1999b; *Salminen* et al., 2004; *Penders* et al., 2006; *Biasucci* et al., 2008, 2010; *Dominguez-Bello* et al., 2010).

Also a differential pattern of Enterobacteriacea is found: *E. coli*, the obligate colonizer in historical data, is less common in C-section infants, while other Enterobacteriacea are more commonly found (*Adlerberth* et al., 2006). A recent systematic review of seven relevant papers concluded that C-section babies had lower abundance and diversity of the phyla Actinobacteria and Bacteroidetes, and higher abundance and diversity of the phylum Firmicutes from birth to 3 months. *Bifidobacterium*, and *Bacteroides* genera were significantly more frequent in vaginally delivered infants compared with C-section delivered (*Rutayisire* et al., 2016). Also noteworthy, the gut microbiota of infants delivered by C-section showed significantly less resemblance to their mothers (*Bäckhed* et al., 2015). This mismatch may be of importance with regard to the foeto-maternal immune cross talk.

With time the C-section babies gut microbiota normalizes and resembles that of the natural delivered babies. The systematic review concluded that by the age of six months the C-section babies had a similar gut microbiota composition as the vaginal delivered babies (*Rutayisire* et al., 2016). However, one study reported that *Bacteroides* colonization was delayed by up to 1 year in caesarean section-delivered compared with vaginally delivered infants (*Adlerberth* et al., 2006), and another study suggests that the differential composition in babies delivered by C-section may be of long-lasting character and still evident at 7 years of age (*Salminen* et al., 2004). The discrepancy may be due to other factors associated with C-section but still varying across studies, such as gestational age, concurrent use of antibiotics and lack of breastfeeding. Yet, it is important to have a better understanding of the duration of the disruptions caused by C-section itself. Even if the gut microbiota is normalized with time, early disruptions may have long-term effects due to the presence of developmental windows that rely on microbial stimulus from the gut, which were mentioned above. If the disruptions are of short durations on the other hand, they may have no long-term effects.

GUT MICROBIOTA IN TERM, BREASTFED, C-SECTION INFANTS, NOT EXPOSED TO ANTIBIOTICS

It is thus unknown to what degree the observed associations between C-section and gut microbiota composition is influenced by concurrent antibiotic use, prematurity, and lack of breastfeeding, since no studies to our knowledge have categorized C-section deliveries according to these factors and studied them separately. For the purpose of the present paper we used the NoMic cohort in Norway, which consists of 524 new-borns and their mothers. Details of this study have previously been published (*Eggesbø* et al., 2011; *White* et al., 2013). We identified term babies, with no concurrent infant use of antibiotics, exclusively breastfed first month and still partially breastfed at 4 months of age, based on maternal reports that were further validated with information in the Norwegian Medical Birth Registry. Altogether 147 infants were identified who fulfilled these criteria. They were further divided into two groups; C-section or normally delivered composition examined. Microbial species and groups have been identified at 4, 10, 30 and 120 days after birth in this study by 23 probes targeting the gene encoding ribosomal RNA

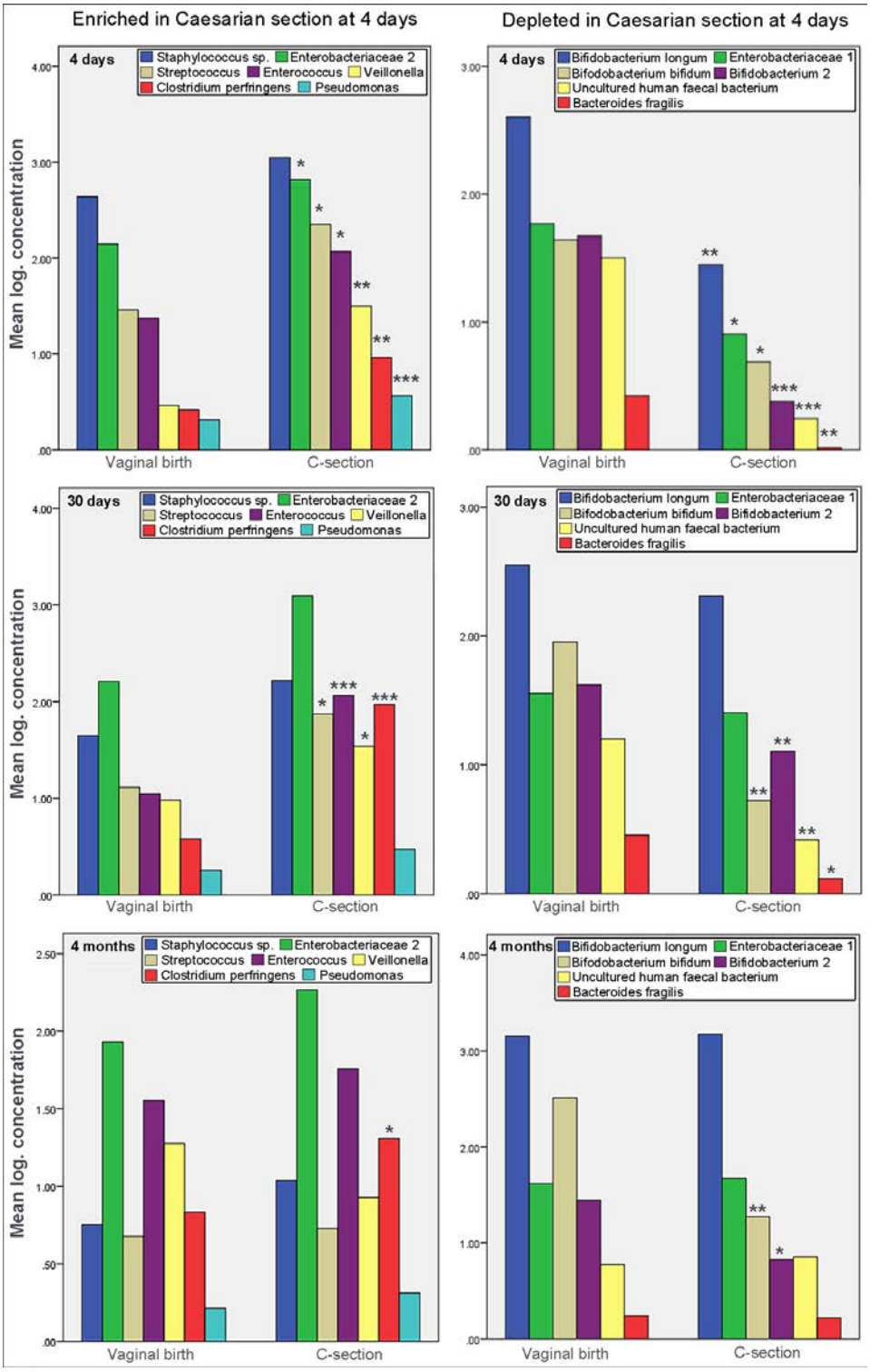

Figure 1: Differences in gut microbiota composition according to mode of delivery, as characterized by means of 22 specific groups (see Table 1) whereby the ones that were significant at day 4 are shown at all time points. *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$.

(16S rRNA) which were developed based on faecal samples from ten children randomly selected among those who had been delivered by caesarean section, and ten children among those who had had a normal delivery in the NoMIC cohort, as previously described (*Rudi* et al., 2007) (Table 1). We observed marked differences in 13 out of the 23 probes signalling various species, with regard to mode of delivery, as shown in Figure 1. Note that signalling cannot be compared across microbial groups (e.g. cannot infer that there is more of microbe A than microbe B), but only across delivery mode and age (e.g. there is more of microbe A in C-section infants compared to vaginal delivery and this microbe increases with age). At 4 months the probes Lachno_Inc_b120lnc and Lachnospira_a120lnc were additionally significantly different across groups. The differences were most marked at day 4, but still marked at one month. C-section delivered infants had significantly higher concentrations of *Streptococcus*, *Enterococcus*, *Veillonella*, *Clostridium perfringens* and *Pseudomonas*, while they had lower concentrations of *Bifidobacterium longum, Bifidobacterium bifidum and Bifidobacterium 2*. *Bacteroides fragilis* was barely detected in C-section infants and also uncultured human faecal bacterium, which represents bacteroides species, was marked lower. At 4 months the differences were less marked, but C-section infants still had more *Clostridium*, and significantly less *Bifidobacteria.*

CONCLUSION

In conclusion, epidemiological studies support an association between C-section and adverse health outcomes, and the underlying mechanism may be due to an altered gut microbiota in C-section delivered babies. Studies indicate that the altered gut microbiota goes beyond the first year of life, thus may be of consequence for developmental windows in early life depending on microbial input.

One of the limitations in current literature is that C-section children have not been characterized with regard to concomitant risk factors. Thus comparing C-section infants across studies is complicated by the fact that the infants may be characterized by highly varying associated risk factors; concomitant disease in mother and/or child, antibiotic use, and varying gestational ages, all factors known to affect gut microbiota composition. We show in this paper that gut microbiota composition is affected by caesarean delivery independent of these factors. We did this by comparing children delivered naturally or by C-section, who were otherwise equal in terms of being breastfed, having received no antibiotics and all being term babies. We see marked differences up to one month of age, and less marked but still significant differences up to four months of age.

Yet, we still do not know which specific properties of the gut microbiota that may play a role in development of disease, nor the timing of the critical windows. Longitudinal cohort studies describing the functions of gut microbiota in children are needed to give further insight into this field.

ACKNOWLEDGEMENT

The research of S.D.P. was supported, in part, by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (Z01 ES101744-04).

LITERATURE

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., and Versalovic, J.: The placenta harbors a unique microbiome. Sci. Transl. Med. 6, 237ra65 (2014).

Adlerberth, I., Carlsson, B., de Man, P., Jalil, F., Khan, S.R., Larsson, P., Mellander, L., Svanborg, C., Wold, A.E., Hanson, L.A.: Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. Acta Paediatr. Scand. 80, 602-610 (1991).

Adlerberth, I., Jalil, F., Carlsson, B., Mellander, L., Hanson, L.A., Larsson, P., Khalil K, and Wold A.E.: High turnover rate of Escherichia coli strains in the intestinal flora of infants in Pakistan. Epidemiol. Infect. 121, 587-598 (1998).

Adlerberth, I., Lindberg, E., Aberg, N., Hesselmar, B., Saalman, R., Strannegard, I.L., and Wold, A.E.: Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? Pediatr. Res. 59, 96-101 (2006).

Amiri, S., Malek, A., Sadegfard, M., and Abdi, S.: Pregnancy-related maternal risk factors of attention-deficit hyperactivity disorder: a case-control study. ISRN Pediatr. 2012, 458064 (2012).

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., AND Gordon, J.I.: Host-bacterial mutualism in the human intestine. Science 307, 1915-1920 (2005).

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M.T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y.S., Kotowska, D., Colding, C., Tremaroli, V., Yin, Y., Bergman, S., Xu, X., Madsen, L., Kristiansen, K., Dahlgren, J., and Wang, J.: Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe 17, 690-703 (2015).

Bager, P., Wohlfahrt, J., and Westergaard, T.: Caesarean delivery and risk of atopy and allergic disease: meta-analyses. Clin. Exp. Allergy 38, 634-642 (2008).

Bennet, R. and Nord, C.E.: Development of the faecal anaerobic microflora after caesarean section and treatment with antibiotics in newborn infants. Infection15, 332-336 (1987).

Bennet, R., Eriksson, M.F., Tafari, N.F., and Nord, C.E.: Intestinal bacteria of newborn Ethiopian infants in relation to antibiotic treatment and colonisation by potentially pathogenic gram-negative bacteria. Scand. J. Infect. Dis. 23, 63-69 (1991).

Biasucci, G., Benenati, B.F., Morelli, L.F., Bessi, E., and Boehm, G.: Cesarean delivery may affect the early biodiversity of intestinal bacteria. J. Nutr. 138, 1796S-1800S (2008).

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., and Retetangos, C.: Mode of delivery affects the bacterial community in the newborn gut. Early Hum. Dev. 86, Suppl 1, 13-15 (2010).

Breton, J., Tennoune, N., Lucas, N., Francois, M., Legrand, R., Jacquemot, J., Goichon, A., Guérin, C., Peltier, J., Pestel-Caron, M., Chan, P., Vaudry, D., do Rego, J.C., Liénard, F., Pénicaud, L., Fioramonti, X., Ebenezer, I.S., Hökfelt, T., Déchelotte, P., and Fetissov, S.O.: Gut Commensal E. coli Proteins Activate Host Satiety Pathways following Nutrient-Induced Bacterial Growth. Cell Metab. 23, 324-334 (2016).

Bullen, C.L., Tearle, P.V., and Willis, A.T.: Bifidobacteria in the intestinal tract of infants: an in-vivo study. J. Med. Microbiol. 9, 325-333 (1976).

Table 1: Probe number, name, labelling sequence and target bacteria according to search in the Ribosomal database project (RDP) database is shown. The table also shows the number of hits in the Palmer clone library (3845 clone sequences).

Probe nr.	Probe name (bacteria)	Target bacteria[a]	Hits in the Palmer clone library (3845 clone sequences)	Labelling probes
1	*Enterococcus*	*Enterococcus* sp. (e.g. *E. faecium*)	44	TCATCCCTTGACGGTATCTAA
2.1	*Lactobacillus* 1	*Lactobacillus* sp. (*L. gassery*, *L. helveticus*, *L. acidophilus*, *L. johnsonii* etc.)	50	GTCAAATAAAGGCCAGTTACTA
3.2	*Lactobacillus* 2a	*Lactobacillus* sp. (*L. casei*, *L. rhamnosous*, *L. rennaquilify*, *L. paracasei*)	1	CAGTTACTCTGCCGACCATT
4.1	*Staphylococcus sp.*	*Staphylococcus* sp.	11	ACACATATGTTCTTCCCTAATAA
5	*Streptococcus*	*Streptococcus* sp.	149	AGTGTGAGAGTGGAAAGTTCA
6	*Clostridium perfringens*	*Clostridium* sp. (*C. perfringens*)	95	TCAACTTGGGTGCTGCATTC
7.5	*Veillonella* 1c	*Veillonella* sp.	49	GATTGGCAGTTTCCATCCCAT
8	*Lachnospiraceae* 1	*Lachnospireaceae* sp. (*Dorea*, *L. Incertea sedis*)	2	AGCTAGAGTGTCGGAGAGG
10	*Lachnospiraceae incertae sedis* 2	*Lachnospireaceae* sp. (*L. Incertea sedis*)	7	TATCAGCAGGAAGATAGTGA
11	*Lachnospiraceae* 2	*Lachnospireaceae* sp. (*Dorea*, *L. Incertea sedis*)	1	AGTCAGGTACCGTCATTTTCT
12.3	*Lachnospiraceae incertae sedis* 3	*Lachnospireaceae* sp. (*L. Incertea sedis*)	1	ACTGCTTTGGAAACTGCAGAT
15.1	*Pseudomonas* 1a	*Pseudomonas* sp.	0	GTCAAAACAGCAAAGTATTAATTTA
15.2	*Pseudomonas* 1b	*Pseudomonas* sp.	0	GTAGAGGGTGGTGGAATTTC
16.1	*Enterobacteriaceae* 1	*Enterobacteriaceae* (*Esherichia coli*, *Shigella*)	221	GAGCAAAGGTATTAACTTTACTC
17	*Enterobacteriaceae* 2	*Enterobacteriaceae* (*Enterobacter* sp.(*Pantoea* sp), *Klebsilla*, *Erwina*, *Citrobacter*, *Bulliauxella*)	696	CGAAACTGGCAGGCTAGAGT
18	Gamma (γ)-Proteobacteria	(γ)-Proteobacteria (*Alteromonadales*, *Vibronales*, *Aeromonadales*, *Enterobacteriales*)	1399	CCTGGACAAAGACTGACGCT
19.1	*Varibaculum*	Varibaculum sp. (*V cambriense*)	3	TTGAGTGTAGGGGTTGATTAG
22	*Bifidobacterium longum*	*Bifidobacterium* (*B. longum*)	114	GAGCAAGCGTGAGTAAGTTTA
23	*Bifidobacterium bifidum*	*Bifidobacterium* (*B. bifidum*)	12	CCGAAGGCTTGCTCCCAAA
24.3	*Bifidobacterium* 1	*Bifidobacterium* (*B. breve*,)	38	CACTCAACACAAAGTGCCTTG
25	*Bifidobacterium* 2	*Bifidobacterium* (*B. thermophilum*, *B. adolescentis*)	7	GCTTATTCGAAAGGTACACTCACCCCGAAGGG
26	*Bacteroides fragilis*	*Bacteriodes* sp. (*B. fragilis*)	1	GGGCGCTAGCCTAACCAG
27.2	Uncultured human fecal bacterium	*Bacteroides* sp.	179	ATGCATACCCGTTTGCATGTA
16S universal	16S rDNA	Bacteria	3710 (96.5%)	CGTATTACCGCGGCTGCTGGCA

[a]Probe Match search (using the labelling probe+ C) in the RDP database (http://rdp.cme.msu.edu/probematch/search.jsp). *One error allowed in search sequence.

Cardwell, C.R., Stene, L.C., Joner, G., Cinek, O., Svensson, J., Goldacre, M.J., Parslow, R.C., Pozzilli, P., Brigis, G., Stoyanov, D., Urbonaite, B., Sipetić, S., Schober, E., Ionescu-Tirgoviste, C., Devoti, G., de Beaufort, C.E., Buschard, K., and Patterson, C.C.: Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies. Diabetologia 51, 726-735 (2008).

Cnattingius, S., Zack, M.F., Ekbom, A.F., Gunnarskog, J.F., Linet, M.F., and Adami, H.O.: Prenatal and neonatal risk factors for childhood myeloid leukemia. Cancer Epidemiol. Biomarkers Prev. 4, 441-445 (1995).

Cook, M.B., Akre, O., Forman, D., Madigan, M.P., Richiardi, L., and McGlynn, K.A.: A systematic review and meta-analysis of perinatal variables in relation to the risk of testicular cancer--experiences of the mother. Int. J. Epidemiol. 38, 1532-1542 (2009).

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

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., and Dinan, T.G.: The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. J. Psychiatr. Res. 43, 164-174 (2008).

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R.: Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. USA 107, 11971-119755 (2010).

Duncan, C.J. and Doyle, J.B.: Cesarean section – A ten-year study of 703 cases at the Boston City Hospital. N. Engl. J. Med. 216, 1-5 (1937).

Ecker, J.L. and Frigoletto, F.D. Jr.: Cesarean delivery and the risk-benefit calculus. N. Engl. J. Med. 356, 885-888 (2007).

Eggesbø, M., Botten, G., Stigum, H., Nafstad, P., and Magnus, P.: Is delivery by cesarean section a risk factor for food allergy? J. Allergy Clin. Immunol. 112, 420-426 (2003).

Eggesbø, M., Botten, G., Stigum, H., Samuelsen, S.O., Brunekreef, B., Magnus, P.: Cesarean delivery and cow milk allergy/intolerance. Allergy 60, 1172117-3 (2005).

Eggesbø, M., Moen, B., Peddada, S., Baird, D., Rugtveit, J., Midtvedt, T., Bushel, P.R., Sekelja, M., and Rudi. K.: Development of gut microbiota in infants not exposed to medical interventions. APMIS 119, 17-35 (2011).

Escherich, T.: Die darmbakterien des neugeborenen und säuglings. Fortschr. Med. 3, 515-522 and 547-554 (1885).

Forsythe, P., Sudo, N., Dinan, T., Taylor, V.H., and Bienenstock, J.: Mood and gut feelings. Brain Behav. Immun. 24, 9-16 (2010).

Gareau, F.E., Mackel, D.C., Boring, J.R., Payne, F.J., Hammett, F.L.: The acquisition of fecal flora by infants from their mothers during birth. J. Pediatr. 54, 313-318 (1959).

Grönlund, M.M., Lehtonen, O.P., Eerola, E., and Kero, P.: Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J. Pediatr. Gastroenterol. Nutr. 28, 19-25 (1999a).

Grönlund, M.M., Salminen, S., Mykkänen, H., Kero, P., and Lehtonen, O.P.: Development of intestinal bacterial enzymes in infants--relationship to mode of delivery and type of feeding. APMIS 107, 655-660 (1999b).

Häger, R., Øian, P., Nilsen, S.T., Holm, H.A., and Berg, A.B.: The breakthrough series on Cesarean section. Tidsskr. Nor. Laegeforen 126, 173-175 (2006).

Hooper, L.V., Xu, J., Falk, P.G., Midtvedt, T., and Gordon, J.I.: A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. USA 96, 9833-9838 (1999).

Hyde, M.J., Griffin, J.L., Herrera, E., Byrne, C.D., Clarke, L., and Kemp, P.R.: Delivery by Caesarean section, rather than vaginal delivery, promotes hepatic steatosis in piglets. Clin. Sci. (Lond.) 118, 47-59 (2010).

Kaye, S.A., Robison, L.L., Smithson, W.A., Gunderson, P.F., King, F.L., and Neglia, J.P.: Maternal reproductive history and birth characteristics in childhood acute lymphoblastic leukemia. Cancer 68, 1351-1355 (1991).

Keag, O., Stock, S., and Norman, J.: Long-term childhood outcomes following caesarean section: Systematic review and meta-analysis. Arch. Dis. Child. Fetal Neonatal Ed. 99, Suppl 1, A8-A9 (2014).

Koplin, J., Allen, K., Gurrin, L., Osborne, N., Tang, M.L., and Dharmage, S.: Is caesarean delivery associated with sensitization to food allergens and IgE-mediated food allergy: a systematic review. Pediatr. Allergy Immunol. 19, 682-687 (2008).

Kühn I, Tullus K, Möllby R.: Colonization and persistence of Escherichia coli phenotypes in the intestines of children aged 0 to 18 months. Infection 14, 7-12 (1986).

Lindberg, E., Nowrouzian, F., Adlerberth, I., and Wold, A.E.: Long-time persistence of superantigen-producing Staphylococcus aureus strains in the intestinal microflora of healthy infants. Pediatr. Res. 48, 741-747 (2000).

Liu, S., Liston, R.M., Joseph, K.S., Heaman, M., Sauve, R., and Kramer, M.S. ; Maternal Health Study Group of the Canadian Perinatal Surveillance System: Maternal mortality and severe morbidity associated with low-risk planned cesarean delivery versus planned vaginal delivery at term. CMAJ 176, 455-460 (2007).

Lotz, M., Gütle, D., Walther, S., Ménard, S., Bogdan, C., and Hornef, M.W.: Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J. Exp. Med. 203, 973-984 (2006).

Lundequist, B., Nord, C.E., and Winberg, J.: The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta Paediatr. Scand. 74, 45-51 (1985).

Ly, N.P., Ruiz-Perez, B., Onderdonk, A.B., Tzianabos, A.O., Litonjua, A.A., Liang, C., Laskey, D., Delaney, M.L., DuBois, A.M., Levy, H., Gold, D.R., Ryan, L.M., Weiss, S.T., and Celedón, J.C.: Mode of delivery and cord blood cytokines: a birth cohort study. Clin. Mol. Allergy 4, 13 (2006).

Mata, L.J., Mejicanos, M.L., Jimenez, F.: Studies on the indigenous gastrointestinal flora of Guatemalan children. Am. J. Clin. Nutr. 25, 1380-1390 (1972).

McAllister, T.A., Givan, J.F., Black, A.F., Turner, M.J., Kerr, M.M., Hutchison, J.H.: The natural history of bacterial colonization of the newborn in a maternity hospital. I. Scott. Med. J. 19, 119-124 (1974).

Midtvedt, A.C. and Midtvedt, T.: Production of short chain fatty acids by the intestinal microflora during the first 2 years of human life. J. Pediatr. Gastroenterol. Nutr. 15, 395-403 (1992).

Midtvedt, A.C., Carlstedt-Duke, B.F., Norin, K.E. FAU, Saxerholt HF, and Midtvedt T.: Development of five metabolic activities associated with the intestinal microflora of healthy infants. J. Pediatr. Gastroenterol. Nutr. 7, 559-567 (1988).

Nelson, D.P. and Mata, L.J.: Bacterial flora associated with the human gastrointestinal mucosa. Gastroenterology 58, 56-61 (1970).

Neufeld, K.A., Kang, N., Bienenstock, J., and Foster, J.A.: Effects of intestinal microbiota on anxiety-like behavior. Commun. Integr. Biol. 4, 492-494 (2011).

Neut, C., Bezirtzoglou, E., Romond, C., Beerens, H., Delcroix, M., and Noel, A.M.: Bacterial colonization of the large intestine in newborns delivered by cesarean section. Zentralbl. Bakteriol. Mikrobiol. Hyg. A 266, 330-337 (1987).

Nowrouzian, F., Hesselmar, B., Saalman, R., Strannegard, I.L., Aberg, N., Wold, A.E., and Adlerberth, I.: Escherichia coli in infants' intestinal microflora: colonization rate, strain turnover, and virulence gene carriage. Pediatr. Res. 54, 8-14 (2003).

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den

Brandt, P.A., Stobberingh, E.E.: Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511-521 (2006).

Rudi, K., Zimonja, M., Kvenshagen, B., Rugtveit, J., Midtvedt, T., and Eggesbø, M.: Alignment-independent comparisons of human gastrointestinal tract microbial communities in a multidimensional 16S rRNA gene evolutionary space. Appl. Environ. Microbiol. 73, 2727-2734 (2007).

Rutayisire, E., Huang, K., Liu, Y., and Tao, F.: The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. BMC Gastroenterol. 16, 86 (2016).

Salminen, S., Gibson, G.R., McCartney, A.L., and Isolauri, E.: Influence of mode of delivery on gut microbiota composition in seven year old children. Gut 53, 1388-1389 (2004).

Simhon, A., Douglas, J.R., Drasar, B.S., and Soothill, J.F.: Effect of feeding on infants' faecal flora. Arch. Dis. Child. 57, 54-58 (1982).

Stark, P.L. and Lee, A.. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. 15, 189-203 (1982).

Steer, P.J. and Modi, N.: Elective caesarean sections--risks to the infant. Lancet 374, 675-676 (2009).

Thavagnanam, S., Fleming, J., Bromley, A., Shields, M.D., and Cardwell, C.R.: A meta-analysis of the association between Caesarean section and childhood asthma. Clin. Exp. Allergy 38, 629-633 (2008).

Thompson, C.L., Wang, B., and Holmes, A.J.: The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2, 739-748 (2008).

White, R.A., Bjornholt, J.V., Baird, D.D., Midtvedt, T., Harris, J.R., Pagano, M., Hide, W., Rudi, K., Moen, B., Iszatt, N., Peddada, S.D., and Eggesbø, M.: Novel developmental analyses identify longitudinal patterns of early gut microbiota that affect infant growth. PLoS Comput. Biol. 9, e1003042 (2013).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 61-68 (2017).

REGULATION OF INTESTINAL BARRIER INTEGRITY BY IgA TARGETED GUT MICROBES IN UNDERNUTRITION

ANDREW L. KAU

Center for Women's Infectious Disease Research,
Washington University School of Medicine, St. Louis, Missouri, USA

SUMMARY

Undernutrition is a leading cause of childhood mortality worldwide. Environmental enteric dysfunction, characterized by recurrent exposure to environmental microbes and subsequent intestinal barrier dysfunction, is increasingly recognized as a major contributor to undernutrition. Analyses of faecal samples from children with undernutrition have documented compositional and functional alterations in their microbiota. IgA is the principal immunoglobulin produced by the gut mucosa. Using fluorescence-activated cell sorting to characterize bacterial taxa targeted by IgA in the gut communities of Malawian twins discordant for severe acute undernutrition (SAM) and then transferring purified IgA+ fractions to germ-free mice, we find that (i) the patterns of microbes targeted by the IgA response in undernourished children differ from that seen in healthy children with increased targeting of Enterobacteriaceae correlating with poorer growth outcomes; (ii) bacterial taxa with bound IgA recovered from SAM donor microbiota can transmit a diet-dependent enteropathy characterized by disruption of the small intestinal and colonic barrier to recipient germ-free mice, and (iii) IgA-targeted microbes present in healthy donor microbiota prevent development of this enteropathy. Together, these results illustrate the diagnostic and therapeutic opportunities provided by defining the gut mucosal IgA responses of children with undernutrition.

INTRODUCTION

The origins of undernutrition encompass both nutritional as well as biological causes. In particular, while there is clearly a role for food insecurity in the genesis of undernutrition, additional factors are likely contributory (*Richard* et al., 2014). Along with poor perinatal nutrition and recurrent acute infections, impairment of gut barrier function has been identified as a major factor contributing to childhood undernutrition (*Trehan* et al., 2016). Defective gut barrier function leads to reduced absorption of nutrients from foods, leakage of host proteins into the intestines and increased inflammation within the gut, leading to additional energy expenditure. The insults leading to impaired intestinal barrier function are not definitely known, however the prominent association of poor sanitation with undernutrition has led to the hypothesis that colonization with certain microbes leads to chronic impairment of the gut barrier (*Humphrey*, 2009; *Keusch* et al., 2014). This syndrome, called environmental enteric dysfunction (EED, formerly tropical

sprue, tropical enteropathy or environmental enteropathy) is thought to contribute greatly to the overall cycle of inflammation, gut functional loss, and undernutrition. Although the association of EED with poor hygienic conditions is well established (*Trehan* et al., 2016), considerably less is known about how perturbations of the gut microbiota may affect the gut barrier function in undernutrition.

GUT MICROBIOTA AND UNDERNUTRITION

Establishing an association between undernutrition and the gut microbiota poses many technical and scientific challenges since genetic, dietary and environmental exposures can all impact gut microbiota structure. In order to circumvent these obstacles, we performed a prospective clinical study on Malawian twins recruited shortly after birth. During study visits faecal microbiota specimens and anthropometric measurements were collected and the children were screened for severe undernutrition. Over the course of the study, 13 twin pairs were determined to be discordant for a form of severe acute undernutrition called kwashiorkor. Analyses of these samples revealed broad differences in the metabolic and functional capacities of these discordant microbiota. Transplantation of the faecal microbiota from the malnourished twin into gnotobiotic mice fed a macro- and micronutrient deficient diet resulted in significantly more weight loss compared to mice colonized with the microbiota originating from the healthy co-twin fed the same diet (*Smith* et al., 2013). These changes were associated with altered metabolic profiles in the recipients of the kwashiorkor microbiota, particularly a reduction in methionine and cysteine, indicating abnormal sulphur metabolism in these animals. Additional analysis of metagenomic data derived from the of the malnourished twins' faecal microbiota demonstrated delayed functional maturation compared to their healthy counterparts (*Smith* et al., 2013).

Alterations in the maturation of the gut microbiota were also observed in a Bangladeshi birth cohort of healthy and malnourished children (*Subramanian* et al., 2014). In this study, a cohort of healthy Bangladeshi children was used to develop a model of microbiota maturation over the first two years of life. Comparison of this model to children with SAM demonstrated a chronological delay in their microbiota maturation (*Subramanian,* et al., 2014). Alarmingly, intervention with a therapeutic diet did not entirely reverse the measured microbiota immaturity, emphasizing the need for new approaches to effect long-term changes in microbiota immaturity (*Subramanian,* et al., 2014).

INTESTINAL BARRIER FUNCTION AND THE MICROBIOTA

Alterations in gut microbiota membership during undernutrition have both short and long term consequences on a child's metabolic, immune and absorptive capacities. It is reasonable to infer that these changes are probably most dramatic at the gut barrier, the site of closest contact between the host and the gut microbiota. The gut intestinal barrier is the series of host defences that allow the peaceful coexistence of our intestinal microbiota and immune

system. These defences are necessarily both complex and overlapping because they must simultaneously maintain a selective partition between some components of the intestinal lumen while allowing the absorption of nutrients and the surveillance of the microbiota by the immune system. To achieve this, an overlapping and multi-tiered "defence in depth" has co-evolved with the microbiota.

First, physical barriers prevent penetration of bacteria through the intestinal epithelium. For example, mucins secreted by goblet cells create a glycoprotein mesh that prevents incursion of bacteria (*McGuckin* et al., 2011). Disruption of the microbiota by antibiotic treatment can trigger thinning of the mucus layer, resulting in increased susceptibility to enteric infection (*Wlodarska* et al., 2011). Additionally, the intestinal epithelium itself regulates the penetration of the intestinal contents into the lamina propria through the modulation of tight junctions between cells (*Groschwitz* et al., 2009). These tight junctions are necessarily malleable because of their critical role in allowing uptake of nutrients from digested foods (*Marchiando* et al., 2010). Second, chemical barriers help to dissuade bacteria from venturing too close to the intestinal epithelium. Antimicrobial peptides released by intestinal epithelial cells as well as Paneth cells segregate the epithelium and gut microbiota (*Vaishnava* et al., 2011). Third, immunologic barriers functioning to prevent, repel and repair insults to the epithelium constitute yet another layer of the intestinal barrier.

Among these immunologic components of the intestinal barrier, immunoglobulin A (IgA) is uniquely suited for studying the interplay between the gut microbiota and intestinal barrier function. IgA is abundant on all mucosal surfaces particularly the gut, where it functions by binding bacteria, toxins and other antigens to prevent them from interacting with the host, a process known as "immune exclusion" (*Stokes* et al., 1975). The synthesis of IgA occurs in response to both innate and adaptive immune signals (*Bunker* et al., 2015). Also, IgA production changes rapidly in response to alterations in the microbiota (*Hapfelmeier* et al., 2010). Thus, identifying which members of the microbiota are targeted by the IgA response will help better elucidate the dynamic changes that occur at the gut barrier in response to diet, infection or breakdown of other components of the gut barrier.

BARRIER INTEGRITY AND THE ROLE OF IgA

We and others have developed a flow cytometry based method for identifying IgA-coated bacteria from faecal samples (*Kau* et al., 2015; *Palm* et al., 2014). Using this approach, which we have termed "BugFACS", we characterized the patterns of IgA targeting in mice colonized with the faecal microbiota of either a Malawian co-twin diagnosed with kwashiorkor, or the co-twin that remained healthy.

Similar to our previous findings (*Smith* et al., 2013), mice colonized with the malnourished microbiota lost more weight than mice colonized with the healthy microbiota fed the same macro- and micronutrient deficient diet. In addition, BugFACS analysis showed that in mice colonized with the malnourished microbiota, there was preferential targeting of members of Enterobacteriaceae. In mice colonized with the healthy co-twin's microbiota, *Akkermansia mucinaphila*, a member

of the phylum Verrucomicrobia, was preferentially targeted. The capacity of the IgA targeted microbes from the malnourished microbiota to induce barrier dysfunction was demonstrated by their ability to induce severe weight loss and mortality when transplanted to another generation of gnotobiotic mice. This barrier dysfunction was associated with mislocalization of the cell adhesion molecule, EpCAM, within the small intestine and epithelial shedding within the large intestine. Interestingly, IgA targeted Enterobacteriaceae alone were insufficient to produce this pathology and required other IgA targeted bacteria, including members of Bacteroidales, to produce overt disease (*Kau* et al., 2015).

The importance of both Bacteroidales and Enterobacteriaceae in undernutrition has also been described in a model of Environmental Enteric Dysfunction (EED) (*Brown* et al., 2015). In this study, oral exposure to the combination of Bacteroidales and *E. coli* resulted in growth stunting accompanied by histopathologic changes consistent with EED including small intestinal villous blunting and disruption in the expression of tight junction genes CLDN2 and CLDN4. Similar to our findings, neither the Bacteroidales nor *E. coli* were capable of inducing barrier dysfunction when administered singly (*Brown* et al., 2015).

Interaction between *E. coli* and Bacteroidales has been noted before in several contexts. In a mixed infection model of abdominal abscess, the presence of *Bacteroides* sp. potentiated abscess formation (*Rotstein* et al., 1989a), possibly mediated through inhibition of neutrophil function by a soluble Bacteroides molecule (*Rotstein* et al., 1989b). Additionally, transcriptional analysis of *E. coli* and *B. thetaiotaomicron* in mono- and bicolonization experiments have shown that these species may crossfeed, potentially enhancing their *in vivo* fitness (*Li* et al., 2015). These data emphasize the need for consideration of bacterial interactions in future experiments directed at understanding the microbiota in modulation of barrier function.

IGA TARGETED BACTERIA IN HUMANS

The ability to determine the targets of the gut mucosal IgA response offers the opportunity to address many important questions regarding the developmental maturation of the gut mucosal barrier in early childhood. In order to tackle some of these questions, we examined a cohort of 40 healthy American twin pairs with faecal samples collected monthly from birth to 2 years of age and performed BugFACS analysis as well as whole community V4-16S rRNA sequencing (*Planer* et al., 2016). Analyses of these data show that IgA responses to the gut microbiota amongst twins were very similar in the first two years of life compared unrelated twins. However, by 2 years the similarity in IgA targeting profiles between unrelated individuals began to converge such that family membership was no longer distinguishable based on IgA profiles. Also, at two years, the children's' IgA profile largely resembled the mothers' implying that IgA mucosal responses have adapted an adult-like configuration by this time. Remarkably, there was a surprising consistency in the most prominent members of the microbiota targeted in both the children and mothers, especially *A. muciniphila* and *Ruminococcus torques*. Both of these taxa were nearly always targeted by the host IgA re-

sponse when present in an individual's faecal sample (*Planer* et al., 2016).

In malnourished children, different taxa predominate amongst the IgA targeted taxa. Consistent with our findings from humanized gnotobiotic mice, members of Enterobacteriaceae were significantly targeted by the IgA response in two cohorts of Malawian children with varying degrees of undernutrition (*Kau* et al., 2015). The degree of IgA targeting appeared to be negatively associated with anthropometric measurements, with individuals having the highest degree of IgA targeting of Enterobacteriaceae also showing the poorest (lowest) weight-for-age and weight-for-height Z-scores. Additionally, the IgA response to Enterobacteriaceae was significantly associated with the presence of virulence factors for enteropathogenic *E. coli* (EPEC) and enteroaggragative *E. coli* (EAEC), likely reflecting mucosal immune activation in response to colonization with these pathogens (*Kau* et al., 2015). Compared to healthy American cohorts (*Planer* et al., 2016), which demonstrated limited targeting of Enterobacteriaceae, the strong IgA responses to Enterobacteriaceae may reflect a compensatory response to the recurrent exposure to these bacteria and could be indicative of a degraded mucosal barrier.

The potential disease-causing role of IgA targeted bacteria is also supported by data from the inflammatory bowel disease literature. Inducing colitis using dextran sulphate sodium, gnotobiotic mice colonized with bacteria found to be targets of the IgA response in patients with inflammatory bowel disease results in severe colonic inflammation. In contrast, colonization with bacteria untargeted by the IgA response from the same microbiota donor displayed only minimal inflammation (*Palm* et al., 2014). When weighed together with our own observations in undernutrition, these studies indicate that IgA targeted intestinal microbes have at least some potential to exacerbate, if not precipitate, intestinal barrier dysfunction.

Perhaps equally interesting, IgA targeted bacteria from healthy individuals may have a role in protecting barrier integrity. Both *A. muciniphila* and *Bifidobacterium bifidum*, identified as targets of the IgA response in children (*Planer* et al., 2016), have both been proposed as probiotics. *B. bifidium* has long been associated with the healthy infant gut microbiota and has more recently been shown to prevent TNF-α mediated gut epithelial barrier disruption *in vitro* (*Hsieh* et al., 2015). Likewise, *A. muciniphila* reduces diet induced metabolic endotoxaemia and its presence correlates improved metabolic parameters of a variety of obesity induced abnormalities including insulin levels, glucose and triglycerides (*Everard* et al., 2013; *Schneeberger* et al., 2015). In our gnotobiotic model undernutrition, we found that *A. muciniphila*, administered with another IgA targeted organism, *Clostridium scindens*, was capable of preventing the barrier dysfunction caused by the administration of IgA targeted microorganisms from an undernourished individuals (*Kau* et al., 2015). These results hint that IgA targeted microbes from healthy individuals may occupy a privileged niche that simultaneously allows prolonged immunological contact with the host while avoiding overt inflammation that would result in degradation of the intestinal barrier. In return, the presence of these "friendly" IgA targets may prevent invasion of harmful taxa, synthesize useful metabolites or provide a basal amount of signalling that promote a healthy gut barrier such as epithelial turn over or mucus secretion.

The factors that result in a bacterial

taxa becoming a target of IgA in the intestine are not known, but will undoubtedly encompass multiple variables. However, one unifying factor for many of the bacteria that we have identified as being targeted by the IgA response is that the lifestyle of these bacteria may bring them in close association with the host epithelium. Both *A. muciniphila* and *R. torques* are known to degrade host mucins (*Derrien* et al., 2004; *Png* et al., 2010) while both EPEC and EAEC are known to adhere to the intestinal epithelium as part of their pathogenic lifecycles (*Kaper* et al., 2004). Alternatively, IgA responses may be driven primarily by bacterial encoded factors, such as capsule (*Peterson* et al., 2015), that modulate antigenicity in response to the environment. Regardless, understanding the features that promote IgA responses in the mucosa is of great practical importance as efforts to rationally select next-generation probiotics for undernutrition will need to take into account their effects on host barrier function.

CONCLUSIONS

Future therapies directed at improving barrier function in undernutrition will rely on a deeper understanding of the microbial and host dynamics at the gastrointestinal barrier. In the absence of biopsy specimens, proxy measurements of barrier function such as identifying IgA targeted bacteria, will greatly aid in our understanding of EED and may constitute a new diagnostic modality for barrier function. Further, efforts to recover IgA-targeted microbes from healthy individuals may lead to next-generation probiotics that favourably modulate barrier function.

LITERATURE

Brown, E.M., Wlodarska, M., Willing, B.P., Vonaesch, P., Han, J., Reynolds, L. A., Arrieta, M.C., Uhrig, M., Scholz, R., Partida, O., Borchers, C.H., Sansonetti, P.J., and Finlay, B.B.: Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. Nat. Commun. 6, 7806 (2015).

Bunker, J.J., Flynn, T.M., Koval, J.C., Shaw, D.G., Meisel, M., McDonald, B.D., Ishizuka, I.E., Dent, A.L., Wilson, P.C., Jabri, B., Antonopoulos, D.A., and Bendelac, A.: Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A. Immunity 43, 541-553 (2015).

Derrien, M., Vaughan, E.E., Plugge, C.M., and de Vos, W.M.: Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol. 54, 1469-1476 (2004).

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G., Delzenne, N.M., de Vos, W.M., and Cani, P.D.: Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc. Natl. Acad. Sci. USA 110, 9066-9071 (2013).

Groschwitz, K.R. and Hogan, S.P.: Intestinal barrier function: molecular regulation and disease pathogenesis. J. Allergy Clin. Immunol. 124, 3-20; quiz 21-22 (2009).

Hapfelmeier, S., Lawson, M.A., Slack, E., Kirundi, J.K., Stoel, M., Heikenwalder, M., Cahenzli, J., Velykoredko, Y., Balmer, M.L., Endt, K., Geuking, M.B., Curtiss, R., 3rd, McCoy, K.D., and Macpherson, A.J.: Reversible microbial colonization of germ-free mice reveals the dynamics of IgA

immune responses. Science 328, 1705-1709 (2010).

Hsieh, C.Y., Osaka, T., Moriyama, E., Date, Y., Kikuchi, J., and Tsuneda, S.: Strengthening of the intestinal epithelial tight junction by Bifidobacterium bifidum. Physiol. Rep. 3, e12327 (2015).

Humphrey, J.H.: Child undernutrition, tropical enteropathy, toilets, and handwashing. Lancet 374, 1032-1035 (2009).

Kaper, J.B., Nataro, J.P., and Mobley, H.L.: Pathogenic Escherichia coli. Nat. Rev. Microbiol. 2, 123-140 (2004).

Kau, A.L., Planer, J.D., Liu, J., Rao, S., Yatsunenko, T., Trehan, I., Manary, M.J., Liu, T.C., Stappenbeck, T.S., Maleta, K.M., Ashorn, P., Dewey, K.G., Houpt, E.R., Hsieh, C.S., and Gordon, J.I.: Functional characterization of IgA-targeted bacterial taxa from undernourished Malawian children that produce diet-dependent enteropathy. Sci. Transl. Med. 7, 276ra224 (2015).

Keusch, G.T., Denno, D.M., Black, R.E., Duggan, C., Guerrant, R.L., Lavery, J.V., Nataro, J.P., Rosenberg, I.H., Ryan, E.T., Tarr, P.I., Ward, H., Bhutta, Z.A., Coovadia, H., Lima, A., Ramakrishna, B., Zaidi, A.K., Hay Burgess, D.C., and Brewer, T.: Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences. Clin. Infect. Dis. 59, Suppl. 4, S207-S212 (2014).

Li, H., Limenitakis, J.P., Fuhrer, T., Geuking, M.B., Lawson, M.A., Wyss, M., Brugiroux, S., Keller, I., Macpherson, J.A., Rupp, S., Stolp, B., Stein, J.V., Stecher, B., Sauer, U., McCoy, K.D., and Macpherson, A.J.: The outer mucus layer hosts a distinct intestinal microbial niche. Nat. Commun. 6, 8292 (2015).

Marchiando, A.M., Graham, W.V., and Turner, J.R.: Epithelial barriers in homeostasis and disease. Annu. Rev. Pathol. 5, 119-144 (2010).

McGuckin, M.A., Linden, S.K., Sutton, P., and Florin, T.H.: Mucin dynamics and enteric pathogens. Nat. Rev. Microbiol. 9, 265-278 (2011).

Palm, N.W., de Zoete, M.R., Cullen, T.W., Barry, N.A., Stefanowski, J., Hao, L., Degnan, P.H., Hu, J., Peter, I., Zhang, W., Ruggiero, E., Cho, J.H., Goodman, A.L., and Flavell, R.A.: Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. Cell 158, 1000-1010 (2014).

Peterson, D.A., Planer, J.D., Guruge, J.L., Xue, L., Downey-Virgin, W., Goodman, A.L., Seedorf, H., and Gordon, J.I.: Characterizing the interactions between a naturally primed immunoglobulin A and its conserved Bacteroides thetaiotaomicron species-specific epitope in gnotobiotic mice. J. Biol. Chem. 290, 12630-12649 (2015).

Planer, J.D., Peng, Y., Kau, A.L., Blanton, L.V., Ndao, I.M., Tarr, P.I., Warner, B.B., and Gordon, J.I.: Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. Nature 534, 263-266 (2016).

Png, C.W., Linden, S.K., Gilshenan, K.S., Zoetendal, E.G., McSweeney, C.S., Sly, L.I., McGuckin, M.A., and Florin, T.H.: Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am. J. Gastroenterol. 105, 2420-2428 (2010).

Richard, S.A., McCormick, B.J., Miller, M.A., Caulfield, L.E., Checkley, W.; MAL-ED Network Investigators: Modeling environmental influences on child growth in the MAL-ED cohort study: opportunities and challenges. Clin. Infect. Dis. 59, Suppl. 4, S255-S260 (2014).

Rotstein, O.D., Kao, J., and Houston, K.: Reciprocal synergy between Escherichia coli and Bacteroides fragilis in an intra-abdominal infection model. J. Med. Microbiol. 29, 269-276 (1989a).

Rotstein, O.D., Vittorini, T., Kao, J., McBurney, M.I., Nasmith, P.E., and Grinstein, S.: A soluble Bacteroides by-product impairs phagocytic killing of Escherichia coli by neutrophils. Infect. Immun. 57, 745-753 (1989b).

Schneeberger, M., Everard, A., Gomez-Valades, A.G., Matamoros, S., Ramirez, S., Delzenne, N.M., Gomis, R., Claret, M., and

Cani, P.D.: Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. Sci. Rep. 5, 16643 (2015).

Smith, M.I., Yatsunenko, T., Manary, M.J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A.L., Rich, S.S., Concannon, P., Mychaleckyj, J.C., Liu, J., Houpt, E., Li, J.V., Holmes, E., Nicholson, J., Knights, D., Ursell, L.K., Knight, R., and Gordon, J.I.: Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. Science 339, 548-554 (2013).

Stokes, C.R., Soothill, J.F., and Turner, M.W.: Immune exclusion is a function of IgA. Nature 255, 745-746 (1975).

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., Benezra, A., DeStefano, J., Meier, M.F., Muegge, B.D., Barratt, M.J., VanArendonk, L.G., Zhang, Q., Province, M.A., Petri, W.A., Jr., Ahmed, T., and Gordon, J.I.: Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature 510, 417-421 (2014).

Trehan, I., Kelly, P., Shaikh, N., and Manary, M.J.: New insights into environmental enteric dysfunction. Arch. Dis. Child.101, 741-744 (2016).

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K., and Hooper, L.V.: The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 334, 255-258 (2011).

Wlodarska, M., Willing, B., Keeney, K.M., Menendez, A., Bergstrom, K.S., Gill, N., Russell, S.L., Vallance, B.A., and Finlay, B.B.: Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect. Immun. 79, 1536-1545 (2011).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 69-76 (2017).

GUT EPITHELIAL REGENERATION AND THE MICROBIOME: DECRYPTING SIGNALS IN THE CRYPT

GIULIA NIGRO, THIERRY PEDRON, and PHILIPPE SANSONETTI

Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur,
Unité INSERM 1202, Paris, France

SUMMARY

The intestinal crypt represents a strategic niche where signals are integrated to regulate epithelial regeneration, due to the presence of stem cells and their associated lineages such as Paneth cells and proliferative cells of the transit-amplifying compartment.

We have selected the intestinal crypt as a model to study microbiota-host cross-talks. Our starting hypothesis was that among the complex assemblage of luminal and mucosal commensal species that compose the microbiota, a limited set may enter into a mutualistic interaction with the cells in the intestinal crypt reflecting a long co-evolution in which the epithelial regenerative apparatus may benefits from microbiota-mediated protection.

We are currently developing a cellular microbiology of the intestinal crypt, particularly in the caecum and the colon where we have identified a "crypt specific core microbiota" (CSCM) that is composed of a restricted set of strictly aerobic, non-fermentative microorganisms whose possible function of crypt "gatekeeper" is currently under study. In this context, we have provided the first evidence that a bacterial product, the peptidoglycan fragment muramyl-dipeptide (MDP), exerts a strong and direct NOD2-dependant cytoprotective effect on intestinal stem cells upon induction of a cytotoxic stress.

THE GUT MICROBIOTA: THE FORGOTTEN ORGAN

The microbiota is a complex ecosystem populated by a diverse community of microorganisms, mainly dominated by bacteria, but also comprised of archaea, fungi, protozoa, and viruses, that inhabit a specific area of the. The most studied is the intestinal microbiota, particularly abundant in the colon, in which Bacteroidetes and Firmicutes represent the dominant phyla.

The microbiota has been defined as a "forgotten organ" (*O'Hara* and *Shanahan*, 2006), playing a central role in health and disease. The microbiota provides protective functions by forming a natural defence barrier against pathogens, by occupying niches and receptors, and by producing colicins. It has metabolic functions such as the fermentation of non-digestible dietary residues, the synthesis of vitamins, and the detoxification of dietary carcinogens (*Sansonetti*, 2008). The microbiota contributes to the regulation of the intestinal barrier, modulating physical properties, such as the composition and the thickness of the mucus layer or the tight junctions between the epithelial cells (*Jakobsson* et al., 2015; *Ulluwishewa* et al., 2011). Moreover,

the gut microbiota interacts with the immune system, stimulating its development and maturation (*Chow* et al., 2010).

The microbiota, as well as bacterial products and bacterial metabolites, provide continuous stimuli to the entire epithelial layer, possibly indirectly affecting stem cells that could sense signals from neighbouring cells responding to bacterial agonists. These signals could influence the survival of stem cells and therefore control both proliferation and regeneration of the whole epithelium. The dialogue between the microbiota and the intestinal cells (immune and epithelial) is mainly due to the expression of innate immune receptors (pattern recognition receptors - PRRs) that recognize conserved bacterial motifs defined as "microbe-associated molecular patterns" (MAMPs) (*Kawai* and *Akira*, 2011).

Principal members of the PRRs are the Toll-like receptors (TLRs) and the nucleotide oligomerization domain receptors (NODs). TLRs are transmembrane proteins located at the cellular plasma membrane or at the endosomal membranes. Instead, the NODs are cytosolic proteins.

Upon MAMPs recognition, PRRs initiate a signalling cascade that usually trigger to an inflammatory response, through the activation of the transcriptional factor NF-κβ responsible of the production of pro-inflammatory cytokines such as IL-1β, IL-6, or IFN-γ. Indeed, in the gut, PRRs seem to be crucial for bacterial-host communications and for maintaining intestinal homeostasis (*Abreu* et al., 2005).

Several studies have shown that bacteria or bacterial motifs are necessary for proper host development not only of the gut but also, for instance, of the immune system. The recognition of commensal microflora by TLRs expressed by epithelial cells is required for intestinal homeostasis (*Rakoff-Nahoum* et al., 2004), while the interaction between fragments of peptidoglycan and NOD1 receptors are necessary and sufficient to induce the genesis of isolated lymphoid follicles (*Bouskra* et al., 2008). One can reasonably hypothesize that stem cells, located in the intestinal crypts, could also express PRRs to recognize bacteria, therefore directly responding themselves to the microbiota. It represents a rare situation in which a differentiating and proliferative epithelium is directly exposed to bacteria, both permanent symbionts and occasional pathogens. One can thus hypothesize that co-evolution of mammals with their gut microbiota has led to a balance, protecting the crypt against microbial insults while maintaining a capacity to sense and integrate microbial signals to convert them into signals boosting epithelial regeneration. We have selected the intestinal crypt as a model to study microbiota-host cross-talks. On one side exploring the possibility that a particular microbiota (i.e., crypt specific core microbiota [CSCM]) is selected to survive in the crypt environment particularly because of its adaptation to the niche environment. Such a CSCM may play a homeostatic role by acting as a gatekeeper, preventing the proliferation of more aggressive symbiotic microorganisms (i.e., pathobionts) (*Chow* and *Mazmanian*, 2010) and pathogens, and by providing optimal signalling to the crypt and its environment.

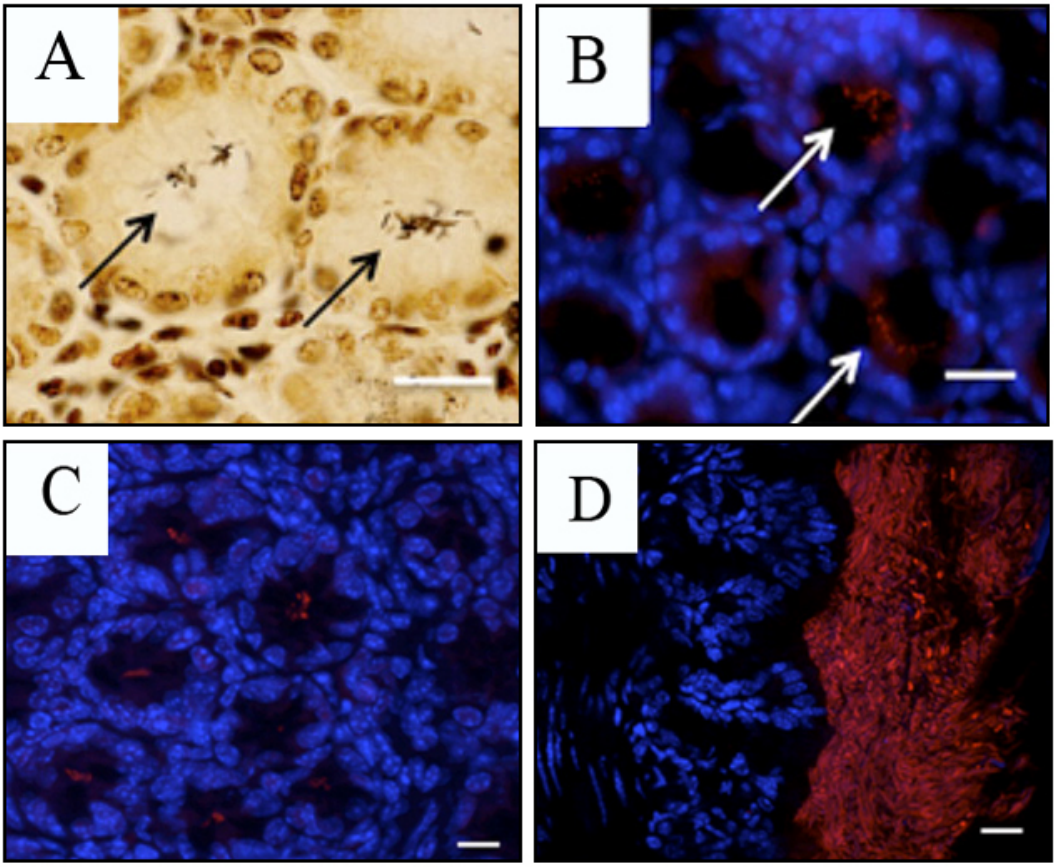

Figure 1: Bacteria reside in the murine proximal colonic crypts (modified from *Pédron* et al., 2012).
A: Warthin-Starry staining, and B: FISH with the universal 16S rRNA gene-targeted probe are shown. Black and white arrows indicate the presence of bacteria. Using specific probes, *Acinetobacter* (C) and the Firmicutes (D) are localized mainly in the crypts and lumen of the colon, respectively.

A CRYPT-SPECIFIC CORE MICROBIOTA RESIDES IN THE MOUSE COLON

Our starting hypothesis was that among the complex assemblage of luminal and mucosal commensal species that compose the microbiota, a limited set may enter into a mutualistic interaction that may reflect a long co-evolution that established a situation in which the epithelial regenerative apparatus may benefit from microbiota-mediated protection.

At stake is the homeostasis of a particularly sensitive zone where adult stem cells are directly exposed to the gut flora.

Preliminary evidence collected from Whartin-Starry (silver/nitrate) staining of various segments of the intestinal tract of various mouse lines reproducibly showed the presence of a small cluster of bacteria located at the crypt bottom in the caecum and proximal colon. These bacteria were not seen in general in the duodeno-jejunum and in the distal colon.

These data were confirmed by fluorescent *in situ* hybridization (FISH) using universal 16S rRNA gene-targeted probe, indicating that these bacteria were alive and metabolically active (Figures 1A and 1B).

We next developed a dedicated pipeline to molecularly identify the relevant bacteria. We combined laser capture microdissection (LCM), DNA amplification with primers flanking the V5-V6 hypervariable regions of 16S rRNA encoding sequences, and 454 sequencing. We clustered the sequences into species-level operational taxonomic units (OTUs) of 97% sequence similarity by the furthest-neighbour method, using the Mothur software program. The analysis was carried-on on mice with different backgrounds and obtained from several providers. We compared samples obtained from luminal and crypt regions and fourteen bacterial phyla were detected, but most sequences could be assigned to five phyla: Firmicutes (73%), Beta- and Gamma-proteobacteria (16%), Actinobacteria (3.5%), and Bacteroidetes (1.7%).

We have compared samples obtained from luminal versus crypt content and we have found that whereas members of the Bacteroidetes were rather poorly represented within both crypt and luminal samples, the Firmicutes represented the majority of luminal sequences (95.5%). The Proteobacteria represented the most abundant sequences found in crypts (47.6%, versus 2.7% for the lumen). Interestingly, the major bacterial family identified was the Moraxellaceae (23.7%), with 23% of *Acinetobacter* spp. sequences in crypts versus 1.6% in the lumen. OTUs from *Acinetobacter* spp. were shared among all crypts, representing a possible common bacterial phylogroup with possible quantitative variations according to the mouse line studied. However, in all cases, levels of *Acinetobacter* spp. in crypts were significantly higher than those observed in luminal samples, in other words a strictly aerobic, non-fermentative genus belonging to the gamma-proteobacterial family.

Thus, using FISH with probes specific for bacterial families and/or genera, the presence of *Acinetobacter* spp. was unequivocally confirmed in crypt samples from different murine strains (more than 10% of the crypts were colonized by *Acinetobacter*, as visualized by FISH) whereas members of the Firmicutes were localized in the lumen (Figures 1C and 1D).

In order to confirm the tropism of *Acinetobacter*, germfree mice were colonized using a conventional microbiota originating from littermates. After 26 days of colonization, bacteria were observed by silver staining in colonic crypts, and the presence of *Acinetobacter* spp. was clearly demonstrated by FISH.

PROTECTIVE EFFECT OF COMMENSALS ON INTESTINAL STEM CELLS

In this context, it was clear that our hypothesis also needed to be validated by demonstrating the existence of a true cross-talk between the microbiota, possibly more specifically the CSCM and the crypt. For this we took advantage from the recently described culture system for intestinal crypts developed by Hans Clever's group: the "miniguts" or "organoids" (*Sato* et al., 2009).

Following purification of murine intestinal crypts and their embedding in

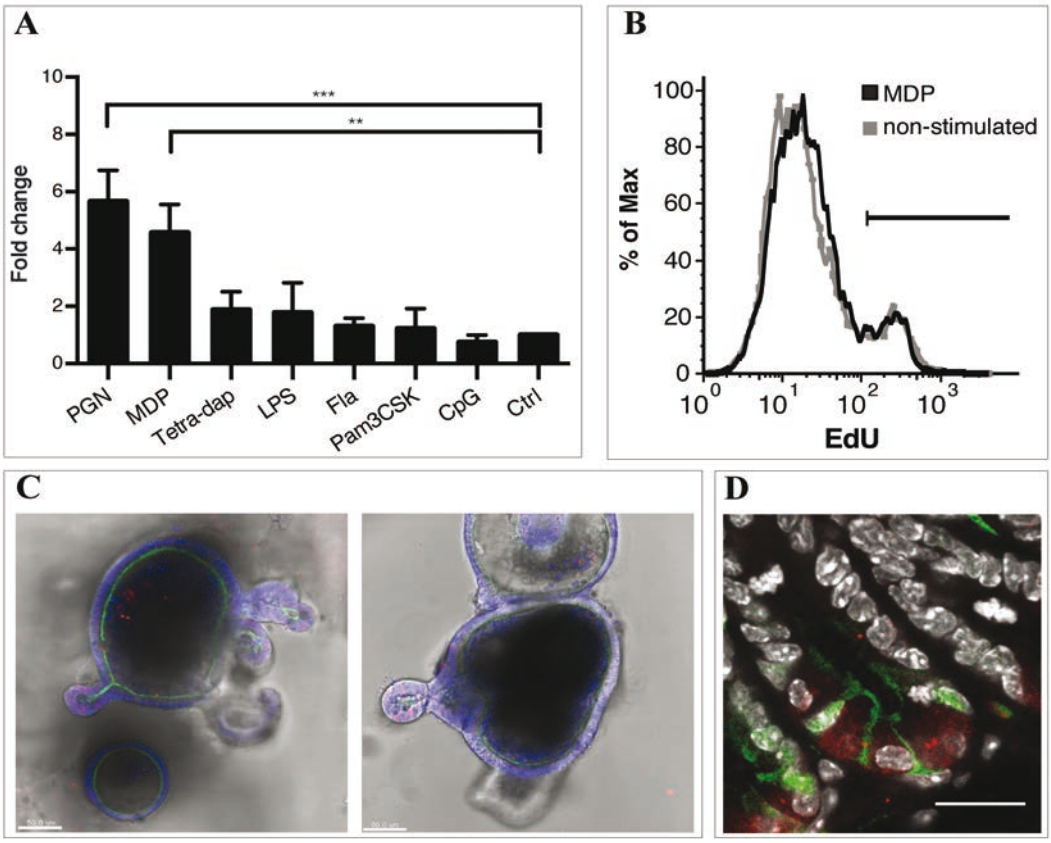

Figure 2: Protective Effect of MDP on intestinal stem cells (modified from *Nigro* et al., 2014).
A: Crypts were stimulated with soluble sonicated peptidoglycan (PGN), muramyl-dipeptide (MDP), muramyl-tetrapeptide (Tetra-dap), *Escherichia coli* lipopolysaccharide (LPS), flagellin (Fla), synthetic lipoprotein (Pam3CSK), or unmethylated CpG dinucleotides (CpG). The fold change in the number of organoids over non-stimulated organoids (Ctrl) was calculated after 4 days of culture.
B: Cell proliferation was analysed by cytometry, monitoring EdU incorporation after 2 h. Representative profiles of MDP-treated (black) and non-treated organoids (grey) are shown.
C: Organoids stimulated (left) or not (right) with MDP were stained with anti-Ki67 (red). Nuclei are in blue and phalloidin in green.
D: The capacity of stem cells to express Nod2 was tested on small intestine sections from Nod2 KO mice. Cells expressing Nod2 are in green, and Paneth cells stained with lysozyme are in red; nuclei are in white.

Matrigel in the presence of essential growth factors such as R-Spondin, Noggin and Wnt ligands, organoids can be grown and maintained *in vitro* showing the progressive appearance of crypt-like structures composed of stem cells, as well as a transit-amplifying compartment followed by cell cycle arrest and differentiation into the various epithelial lineages: Paneth cells in close apposition to stem cells, bona fide epithelial cells, goblet cells and entero-endocrine cells (*Sato* et al., 2009). The organoids will form a three-dimensional structure, recapitulating the crypt-villus architecture, with an internal lumen.

When crypts were exposed, before embedding, to bacterial MAMPs (microbe-associated molecular patterns),

such as peptidoglycan (PGN) muramyl-dipeptide (MDP), muramyl-tri and tetrapeptide, lipopolysaccharide (LPS), flagellin (Fla), unmethylated CpG dinucleotides (CpG), and lipoproteins (Pam3CSK), only crypts exposed to PGN and MDP yielded 4-5 fold more organoids compared to not stimulated organoids (Figure 2A).

To show if the growth rate is affected by the presence of the MDP, all the organoids were imaged and their area measured. No difference was observed in the maximum size of organoids compared to controls, suggesting that stimulation with MAMPs did not affect the growth rate. To confirm this observation, treated and control organoids were tested after 4 days of culture for both EdU incorporation and Ki67 staining (Figures 2B and 2C). We did not observe any variation in the cell proliferation rates between organoids stimulated with MDP and controls, either globally or specifically in the transit-amplifying compartment. Therefore, epithelial proliferation was not affected by MDP stimulation. Moreover, we repeated the *in vitro* experiment of stimulation by using crypts from NOD1KO and NOD2KO mice and we observed the same increase in the yield only in the NOD1KO organoids, indicating that the observed phenotype is linked to NOD2 expression. Further experiments indicated that Lgr5+/CD24middle+ crypt cells corresponding to the stem cells expressed high levels of NOD2 transcripts, the intracellular cytosolic sensor involved in MDP recognition (Figure 2D). Using co-culture of single intestinal stem cells and Paneth cells from wildtype (wt) and/or NOD2KO mice, we demonstrated that this effect was due to the expression of NOD2 in stem cells and not in Paneth cells. To better evaluate the cytoprotective effect of MDP on the stem cells, we first performed *in vivo* experiments on mice in which the microflora was depleted by antibiotic treatment. We showed that mice gavaged with MDP were protected from the effects of doxorubicin, a DNA-intercalating agent that induces high levels of oxidative stress. To test the existence of a NOD2-dependent pathway of stem cell cytoprotection in the presence of microbiota-produced MDP, we carried out similar experiments in conventional wt and NOD2KO mice. We observed that wt mice, not NOD2KO mice, were able to regenerate the gut upon treatment with doxorubicin. Moreover, the wt mice presented higher numbers of crypt survival compared to NOD2KO mice, indicating a protective effect of NOD2. We also showed that crypts extracted from doxorubicin-treated mice were much more responsive to MDP regarding the yields of organoids, thereby indicating that the MDP-NOD2 pathway is rather protective than following cytotoxic aggression in homeostatic conditions. This altogether provides strong support to the concept of a protective cross-talk between the microbiota and the regenerative apparatus of the crypt with particular targeting to the stem cells.

CONCLUSION

Our approach has attempted to set the basis for a cellular microbiology of the mutualistic symbiosis established between elements of the intestinal microbiota and the gut mucosal tissues. We have shown that the caecal and colonic crypts harbour resident microbiota in the mouse and this bacterial population

is unexpectedly dominated by aerobic genera.

Interestingly, this microbiota resembles the restricted microbiota found in the midgut of invertebrates. Thus, the presence of our so-called "crypt-specific core microbiota" in the mouse colon potentially reflects a co-evolutionary process under selective conditions that can now be addressed. We suggest that CSCM could play both a protective and a homeostatic role within the colon.

Our central hypothesis is that the CSCM may act as a crypt "gatekeeper" with multiple complementary functions selected by the co-evolutionary process:

(i) Protection against the intrusion of pathogens or pathobionts that may disturb the fragile homeostasis required to preserve the balance of epithelial regeneration in physiological conditions or following a cytotoxic aggression, and "buffering" of inflammation that may be transiently caused by the accidental passage of a pro-inflammatory pathobiont.

(ii) Biodegradation of the xenobiotic molecules that may gain access to the crypt and induce strong genotoxic damage, particularly on stem cells.

It must be noticed that the CSCM isolates are typical environmental microorganisms with strong and diverse biodegradative activities, as assessed by the annotation of their genomic sequences (*Saffarian* et al., 2015).

Therefore, in the presence of stress, such as doxorubicin, the stem cells are more prone to respond to the MDP released by the microflora that enhances their protection from injury. This work highlighted a new role for NOD2 in intestinal homeostasis. In a steady-state condition, the bacteria perhaps do not give any specific advantage to stem cells, as indicated by the fact that NOD2KO mice are viable and do not present any particular difference compare to wt mice. However, upon injury, the presence of the microbiota and particularly the released MDP, has a protective effect on stem cells, making them more reactive to MDP itself and more resistant to death.

LITERATURE

Abreu, M.T., Fukata, M., and Arditi, M.: TLR signaling in the gut in health and disease. J. Immunol. 174, 4453-4460 (2005).

Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I.G., and Eberl, G.: Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507-510 (2008).

Chow, J., Lee, S.M., Shen, Y., Khosravi, A., and Mazmanian, S.K.: Host-bacterial symbiosis in health and disease. Adv. Immunol. 107, 243-274 (2010).

Chow, J. and Mazmanian, S.K.: A pathobiont of the microbiota balances host colonization and intestinal inflammation. Cell Host Microbe 7, 265-276 (2010).

Jakobsson, H.E., Rodríguez-Piñeiro, A.M., Schütte, A., Ermund, A., Boysen, P., Bemark, M., Sommer, F., Bäckhed, F., Hansson, G.C., and Johansson, M.E.V.: The composition of the gut microbiota shapes the colon mucus barrier. EMBO Rep.16, 164-177 (2015).

Kawai, T. and Akira, S.: Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637-650 (2011).

Nigro, G., Rossi, R., Commere, P.H., Jay, P., and Sansonetti, P.J.: The cytosolic bacterial peptidoglycan sensor Nod2 affords stem cell protection and links microbes to gut epithelial regeneration. Cell Host Microbe 15, 792-798 (2014).

O'Hara, A.M. and Shanahan, F.: The gut flora

as a forgotten organ. EMBO Rep. 7, 688-693 (2006).

Pédron, T., Mulet, C., Dauga, C., Frangeul, L., Chervaux, C., Grompone, G., and Sansonetti, P.J.: A crypt-specific core microbiota resides in the mouse colon. mBio. 3, e00116-12 (2012).

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R.: Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118, 229-241 (2004).

Saffarian, A., Mulet, C., Naito, T., Bouchier, C., Tichit, M., Ma, L., Grompone, G., Sansonetti, P.J., and Pédron, T.: Draft Genome Sequences of Acinetobacter parvus CM11, Acinetobacter radioresistens CM38, and Stenotrophomonas maltophilia BR12, Isolated from Murine Proximal Colonic Tissue. Genome Announc. 3, e01089-15 (2015).

Sansonetti, P.J.: Host-bacteria homeostasis in the healthy and inflamed gut. Curr. Opin. Gastroenterol. 24, 435-439 (2008).

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H.: Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262-265 (2009).

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C.: Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769-776 (2011).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 77-91 (2017).

MICROBIOME-HOST INTERACTIONS THROUGH THE ARYL HYDROCARBON RECEPTOR

SVEN PETTERSSON

The LKC School of Medicine, Nanyang Technological University, Singapore, and Department of Microbiology, Cell and Tumor Biology, Karolinska Institute, Stockholm, Sweden

SUMMARY

The vertical transmission of the maternal microbiome to the new offspring sparks the development of a new microbiome that together with its new host forms a symbiotic entity. The microbial genome (biont) and the genome of the host (biont) represent two "bionts" which together represent a holobiont. The holobiont represent a functional unit with evolutionarily designed functions to secure nutritional intake, DNA replication and reproduction. It follows that virtually all organs within the holobiont respond to changes within the microbiome. The microbiome, in turn reciprocate, by influencing host genome function to support host physiology. The aryl hydrocarbon receptor (AhR) is an evolutionarily conserved receptor recognizing environmental compounds, including natural ligands some of which are derived from the microbiome. This small overview touches upon aspects of the microbiome-AhR communication to display an example of holobiont communication.

INTRODUCTION

Until very recently, evolutionary genetics focused almost exclusively on patterns of variation found within our mitochondrial and nuclear genomes. Yet, bacteria and archaea, two of the predominant kingdoms within the microbiome, were the dominant forms of life on Earth for approximately 3 billion years prior to the evolution of the animal kingdom (*Maloof* et al., 2010; *Bell* et al., 2015). Further to this point, it has become increasingly clear that the study of evolution is not complete without consideration of the microbiome. In addition to the somatic cells of the host, the entire body is a patchwork landscape home to thousands of different microbial species that number in the tens of trillions of cells. Rather than mere transient microbes, these co-resident organisms contain an immense diversity of genes that interact directly with our physiology to carry out vital functions. The awareness of these roles has resulted in a radical shift from thinking of host-associated microbes solely in terms of pathogens, to considering them essential members of host biology important for life. As such, the host-associated microbial communities serve as accessory genetic reservoirs that respond to changes in our environments and lifestyles to converge as a shared target for natural selection.

These new findings represent a paradigm shift in our current understanding of host physiology and place the microbiome into homeostasis mechanisms relevant to digestion and energy metabolism, immune development, neuro-

logical function, and infectious disease susceptibility. The two entities, the microbial genome and the host genome, form a holobiont with overlapping biological and biochemical needs which influence all aspects host physiology. Environmental changes affect both the host and its microbiome. The last two decades of genome-wide association studies has ignored the microbiome and, consequently, missed the response elicited within it. Through the use of germ-free (GF) mice, mice that are raised without exposure to any microbes, the holobiont physiology has begun to be addressed using a systems biology approach (*Nicholson* et al., 2012). This includes the ability of the host and microbiome to communicate, to maintain homeostasis and act correspondingly when exposed to assaults. Furthermore, the use of GF mice helps us to answer fundamental questions about the role of microbial evolution and ecology in broader patterns of host evolution. This mini review focuses on host microbiome interactions by discussing the AhR receptor as an example of an evolutionarily conserved signalling pathway that act as host receiver and transmitter to the microbiome within the holobiont.

A MICROBIOME RESPONDING RECEPTOR WITHIN THE HOLOBIONT; THE ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AhR) is a cytoplasmic ligand-induced receptor originally discovered as a xenobiotic sensor mediating the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), also known as dioxin (*Denison* and *Nagy*, 2003; *Hu* et al., 2007; *Linden* et al., 2010; *Murray* et al., 2014). The metabolism of xenobiotic compounds is initiated by activation of the AhR, which then translocate to the nucleus, where it acts as a transcription factor for specific target genes, such as cytochrome P450 1A1 and cytochrome P450 1B1 (*Ma* et al., 2001; *Denison* and *Nagy*, 2003; *Diani-Moore* et al., 2010; *Linden* et al., 2010; *Opitz* et al., 2011; *Nguyen* et al., 2013; *Stockinger* et al., 2014). However, invertebrates do not have a toxic response to dioxin, and none of the currently known invertebrate AhR orthologues, including in spineless Drosophila, have dioxin binding capacity, which suggests that the ancestral role of the AhR is not specifically a toxin response (*Hahn* et al., 1997; *Hahn*, 2002). Furthermore, physiological roles of the AhR in responses to endogenous ligands have been reported in cell cycle regulation, cell differentiation, and immune responses (*Fernandez-Salguero* et al., 1995; *Benedict* et al., 2000; *Quintana* et al., 2008; *Opitz* et al., 2011; *Hao* and *Whitelaw*, 2013). A number of endogenous AhR ligands have been suggested through *in silico* research and biological testing, including tryptophan metabolites (*Denison* and *Nagry*, 2003; *Nguyen* and *Bradfield*, 2008; *Opitz* et al., 2011). Recently, our group discovered that AhR expression is attenuated in GF mice (*Korecka* et al., 2016). This finding suggests that the AhR acts as a mediator in communication between the host and the microbiome.

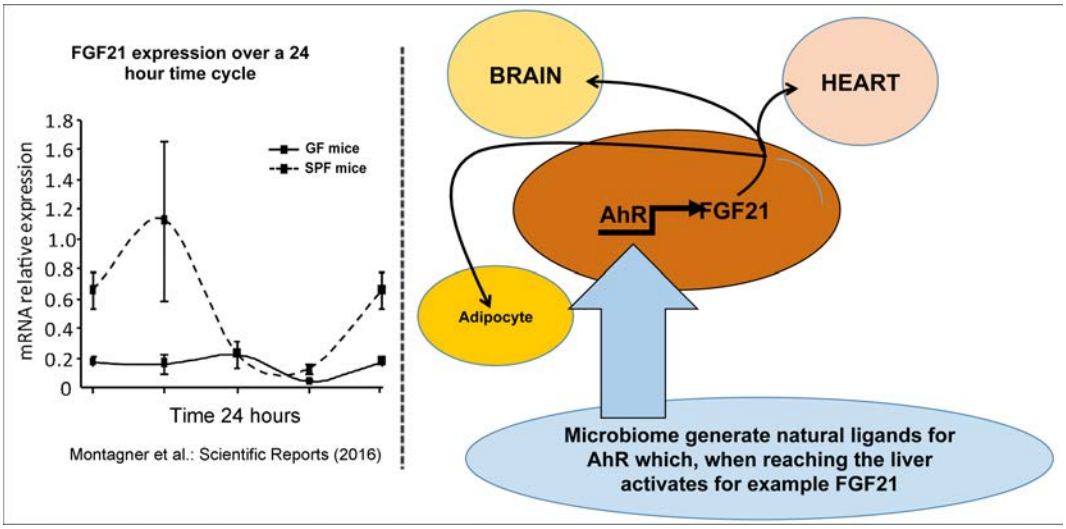

Figure 1: An example of a microbiome-host interaction in the holobiont. Microbiome mediated regulation of AhR and expression of FGF21, a liver secreted inteerorgan communicator, through te microbiome.

THE ARYL HYDROCARBON RECEPTOR RESPONDING OUTPUTS IN THE HOLOBIONT

Dioxin-activated AhR attenuates lipid metabolism via negative regulation of peroxisome proliferator-activated receptor (PPAR) (*Remillard* and *Bunce*, 2002). Dysregulation of lipid metabolism leading to hepatic steatosis and insulin resistance suggests that the AhR plays an important role in integrating exogenous and endogenous influences in lipid and energy metabolism (*Lee* et al., 2010; *Lu* et al., 2015). Findings from AhR-deficient mice show that, like GF mice (*Backhed* et al., 2007; *Rabot* et al., 2010), they are protected from high fat diet-induced obesity, hepatic steatosis, and insulin resistance (*Xu* et al., 2015).

Recently, fibroblast growth factor 21 (FGF21) was reported to be a novel target gene of the AhR. FGF21 increases lipid oxidation and ketogenesis but decreases gluconeogenesis at the gene expression level (*Badman* et al., 2007; *Inagaki* et al., 2007). As an insulin sensitizer, FGF21 boosts the metabolic benefits such as improved blood glucose levels due to increased glucose uptake in adipocytes, reduced body weight due to increased energy expenditure, and improved blood lipid profiles due to hepatic sequestration of lipid droplets (*Kharitonenkov* et al., 2005; *Coskun* et al., 2008; *Lu* and *Klaassen*, 2011). TCDD-induced AhR activation has been shown to increase FGF21 mRNA in both a dose- and time-dependent manner in mouse liver (*Lee* et al., 2010; *Lu* et al., 2015). In addition, drug-induced over-expression of human AhR in mice induces the activation of FGF21, which may then result in decreased insulin resistance (*Cheng* et al., 2014). The opposite effects were observed with the down-regulation of FGF21 – insulin insensitivity, deranged lipid profile, and liver inflammation – and can be associated with the attenuation of hepatic lipid accumulation and increased transfer of fats out of the liver in hepatocyte-tar-

geted AhR knockout (KO) (*Lu* et al., 2015).

Recent work from our lab linked the mechanism of microbiota and host communication through an AhR-dependent mechanism. We demonstrated that the AhR is differentially expressed in GF mice and that the FGF21 expression in liver is subject to regulation by the microbiome (Figure 1). This suggests a microbiome mediated signalling pathway in which microbes regulate FGF21 expression and thus its effector function as an inter-organ communicator (Figure 1). Similarly, our AhR-KO study showed that AhR regulates a set of metabolic genes in the liver, including CD36 (involved in fatty acid uptake) and Hmgcs2 (an enzyme involved in ketone body regulation) (*Korecka* et al., 2016). Similar to fast-induced adipose factor-KO mice (*Backhed* et al., 2007), AhR-KO mice gain weight as expected but do not develop insulin resistance (*Korecka* et al., 2016), suggesting that AhR could be the upstream link between microbiota-mediated signals and the host (*Korecka* et al., 2016).

Several reports have associated AhR function with the regulation of the immune system. TCDD treatment has shown that AhR has the capacity to mediate the differentiation and/or function of T cells, macrophages, and dendritic cells (*Veldhoen* et al., 2008, 2009; *Kimura* et al., 2009; *Ghandi* et al., 2010; *Quintana* et al., 2010; *Hao* and *Whitelaw*, 2013; *Nguyen* et al., 2013; *Stockinger* et al., 2014). The activation of AHR by TCDD (*Warren* et al., 2000; *Vorderstrasse* et al., 2003; *Jin* et al., 2014a) and the ablation of AhR in KO animals (*Yamada* et al., 2016) have implicated this receptor in viral immunity. We also recently reported that ablating the AhR in $CD11c^{+}$ cells perturbs the development of the intestinal epithelium and intestinal immunity (*Chng* et al., 2016). Depending on the presence of specific ligands, AhR activation has also been shown to suppress or exacerbate responses in experimental autoimmune disease models. For example, TCDD and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) can suppress experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS) (*Quintana* et al., 2010), whereas the activation of AhR by ligands such as 6-formylindolo[3,2-b]carbazole (FICZ) exacerbates the development of EAE (*Quintana* et al., 2008, 2010; *Veldhoen* et al., 2008; *Duarte* et al., 2013). In addition, the affinity of AhR for ligands (TCDD, high affinity; FICZ, low affinity) influenced the amount of IL-17 and IL-22 protein secreted by Th17 cells (*Mezrich* et al., 2010). These findings indicate that various ligands for AhR may have different effects on host development.

MICROBIOME DERIVED NATURAL LIGANDS FOR THE ARYL HYDROCARBON RECEPTOR

Though most research on AhR has focused on man-made high affinity binding ligands and chemical pollutants, recent research has implicated important roles for an array of low affinity natural ligands produced, metabolized, or influenced by the gut microbiota. Natural ligands for AhR can be divided into three groups: host mediated, microbiota mediated, and dietary compounds.

The essential amino acid tryptophan is the major source for both host-mediated and microbiome-mediated AhR ligands. Kynurenine (KYN) is converted from tryptophan by tryptophan

2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) and is an important AhR ligand (*Mezrich* et al., 2010). Kynurenic acid (KYNA) is converted from KYN by kynurenine aminotransferase and also an important ligand (*DiNatale* et al., 2010). Our research has shown that the microbiota regulates the expression of IDO in the liver, and although IDO may play a more important role in KYN metabolism in extra-hepatic tissue (*Badawy*, 2015), these results indicate a need to analyse the role of the microbiota in KYN metabolism (*Korecka* et al., 2016).

Gut microbiota also convert tryptophan to indole, indole-3-acetate, and tryptamine, which have been identified in mouse and human intestine and work as AhR agonists and antagonists (*Jin* et al., 2014b; *Hubbard* et al., 2015). Microbial pigment virulence factors, namely the phenazines from microbes such as *Pseudomonas aeruginosa* and the naphthoquinone phthiocol from *Mycobacterium tuberculosis*, act as microbiota-mediated AhR ligands. Upon ligand binding, activation leads to virulence factor degradation and regulates cytokine and chemokine production (*Moura-Alves* et al., 2014). Short chain fatty acids, such as propionic acid and butyrate, from the microbiome are not direct ligands for AhR, but our recent data suggest that they stabilize AhR, increasing its activity in the presence of true ligands. The majority of dietary AhR ligands are produced by plants. Plant-derived compounds that act as ligands for AhR include flavonoids, stilbenes, carotenoids, and some indoles. Indole-3-carbiol (I3C) is an indole compound found in cruciferous vegetables that is converted to higher affinity AhR ligands, such as indolo-[3,2-b]-carbazole and 3,3'-diindolymethane in the acidic environment of the stomach (*Shertzer* and *Senft*, 2000).

MICROBIOME COMMUNICATING WITH THE HOST NERVOUS SYSTEM

Sudo et al. (2014) first demonstrated a possible link between the hypothalamic-pituitary-adrenal (HPA) axis and the gut microbiome. Elevated adrenocorticotropic hormone and corticosterone levels were observed in GF mice compared to specific pathogen-free (SPF) mice in early life. They also demonstrated that brain-derived neurotrophic factor (BDNF) is significantly reduced in the hippocampus and cortex of GF mice (*Sudo* et al., 2014). Later studies confirmed regulation of steady state levels of BDNF by the microbiome (*Bercik* et al., 2011; *Diaz Heijtz* et al., 2011), which plays an important role in neuroplasticity, neuron differentiation, and the maintenance and protection of neurons under stress. Many of these groups have linked changes in brain biochemistry to altered behaviours in GF mice (*Bercik* et al., 2011; *Diaz Heijtz* et al., 2011; *Sudo* et al., 2014).

Recently there have been reports that the microbiome plays an important role in the growth and function of CNS cell populations. Hippocampal neurogenesis was shown to be increased in GF mice (*Ogbonnaya* et al., 2015), which also correlated to increased volume and abnormal neuronal morphology in the hippocampi of GF mice (*Luczynski* et al., 2016). Similarly there was increased amygdala volume in GF mice with concomitant neuronal morphology (*Luczynski* et al., 2016). In contrast to this, hippocampal neuro-

genesis was shown by Möhle and colleagues to be decreased in mice treated with antibiotics (*Möhle* et al., 2016). They demonstrated that their model of antibiotic depletion lead to decreased hippocampal neurogenesis through modulation of the populations of specific immune cells (*Möhle* et al., 2016). Our group have also reported that the microbiome plays a key role in the maintenance of other synaptic proteins, including synaptophysin and PSD-95, which are reduced in the striatum of SPF mice, suggesting abnormally hyperactive synaptogenesis in the striatum of GF mice (*Diaz Heijtz* et al., 2011). The microbiome has also been implicated in the functionality of glial cells. Our group has demonstrated that the microbiota is instrumental in the development of the blood-brain barrier (BBB) (*Braniste* et al., 2014). Finally, *Hoban* et al. (2016) demonstrated that in the absence of microbiota, there is increased myelination of neurons in the pre-frontal cortex. Taken together, these early correlative findings highlight the ability of the microbiome to communicate with the host on critical aspects of host function and further underscore the necessity of a bilateral crosstalk between the microbiome and the host to generate a functional holobiont. That said, the knowledge on underlying molecular mechanisms linking the microbiome to the nervous system remains limited and is what the field needs.

THE ARYL HYDROCARBON RECEPTOR AND THE CENTRAL NERVOUS SYSTEM

Reports on the AhR in neurodevelopment are very limited. However, the AhR appears to be vital in the maintenance of some key pathways in neurodevelopment in worms. In *Caenorhabditis elegans*, *Huang* et al. (2004) demonstrated a role for AhR in neural cell fate determination, particularly for GABAergic neurons. AhR-1 is the AhR orthologue in *C. elegans*. In worms with AhR-1 mutations, two specific neurons out of the 302 total neurons have been reported to appear and act like a second pair of neurons that could be reprogrammed into the first pair of neurons by ectopic administration of AhR-1 (*Huang* et al., 2004). In addition, *Qin* et al. (2004) found that AhR-1 is responsible for the development, orientation, and axonal migration of AhR-1-expressing neurons in *C. elegans*. Taken together, these results demonstrate that AhR contributes to the cell fate determination of specific neuronal populations in worms, possibly through natural ligands and irrespective of dioxin exposure.

Dioxin toxicity studies have demonstrated that the AhR is likely to play a role in CNS development. In zebrafish, TCDD exposure was reported to reduce the total number of neurons by 30 % (*Hill* et al., 2003). In mice, dioxin toxicity studies have demonstrated a similar role for AhR in the embryonic differentiation of GABAergic neurons in the telencephalon (*Gohlke* et al., 2009) and the neurogenesis of cerebellar granule cells (*Williamson* et al., 2005). Importantly, due to the extraordinarily high binding affinity of dioxin for the AhR, emphatic conclusions regarding the physiological role of the AhR in normal development cannot be drawn from dioxin studies alone.

The AhR was also shown to play a crucial role in CNS development in studies more consistent with typical biology. The expression of a constitutively active AhR in mice retarded the

development of interneurons in the olfactory bulb (*Kimura* et al., 2016). Furthermore, in mouse primary cortical neurons, AhR activation by FICZ was also shown to increase the expression of synaptophysin and SAP102, but not PSD95 (*Hsu* et al., 2014). In functional experiments, the AhR was shown to alter hippocampal neurogenesis and contextual fear memory in mice (*Latchney* et al., 2013), as well as aggression behaviour in *C. elegans* (*Qin* et al., 2006). *Latchney* et al. (2013) demonstrated that adult AhR-KO mice and TCDD-exposed mice hippocampal-dependent memory impairment. AhR-deficient mice and TCDD-exposed mice also exhibited reduced cell proliferation, survival, and differentiation in the adult dentate gyrus. The conflicting data demonstrating both the KO and activation of AhR lead to similar outcomes, suggesting that the AhR plays a vital role in CNS homeostasis.

A DYSFUNCTIONAL HOLOBIONT IN EARLY LIFE

Autism spectrum disorder (ASD) is a neurodevelopmental illness for which evidence supports a possible link between the maternal/early postnatal microbiome and dysfunctional neurodevelopmental programming. From a human health perspective, the association between the microbiome and neurodevelopment was highlighted by evidence that people suffering from ASD also frequently present with problems related to a dysfunctional bowel with aberrant intestinal barrier function (*Rosenfeld*, 2015). Although the association remains controversial, a role for dysfunctional gut microbiome-brain axis has gained further support from the recent demonstration of different microbiome composition in children with ASD compared to age-matched controls (*Krajmalnik-Brown* et al., 2015).

A recent study demonstrated that, in an animal model of ASD, correction of the microbiota with probiotic administration of *Bacteroides fragilis* corrected biochemical and behavioural abnormalities associated with ASD (*Hsiao* et al., 2013). In this ASD mouse model, the key effector in the microbiota-gut-brain axis was the metabolome; a number of specific metabolites altered in the ASD mouse model were normalized by the treatment. Indolepyruvate, a microbially controlled molecule that is metabolized into an AhR agonist, was significantly regulated in the ASD model and by *B. fragilis* treatment (*Hsiao* et al., 2013). This metabolite is an interesting corollary to indolyl-3-acryloylglycine, which has been shown to be elevated in the urine of humans with ASD (*Bull* et al., 2003).

Epidemiological studies of Vietnamese children exposed to TCDD in the prenatal and perinatal period have demonstrated increased neurodevelopmental defects and autistic traits in children with greater exposure to TCDD (*Tran* et al., 2016). Prenatal and postnatal exposure to KYN in rats causes cognitive defects in adulthood (*Pocivavsek* et al., 2012). Although Pocivavsek and colleagues did not identify a specific mechanism underlying the association between early life KYN exposure and cognitive deficits, they did note that the treatment led to 3.4- and 2.1-fold increases in KYNA levels in the brain at postnatal days 2 and 21, respectively (*Pocivavsek* et al., 2012). Although they noted the effects of KYNA as an antagonist of the α7 nicotinic acetylcholine receptor and the N-methyl-d-aspartate receptor (*Poci-*

vavsek et al., 2012), KYNA is also an AhR ligand with a stronger binding affinity for the AhR than KYN (*DiNatale* et al., 2010), potentially implicating AhR activity in the cognitive abnormalities observed in this model. While still in its infancy, it is tempting to speculate that the AhR signalling pathway and its microbially derived natural ligands are of great interest to better understand the underlying mechanisms of Autism Spectrum Disorders.

THE HOLOBIONT AND THE BLOOD-BRAIN BARRIER

The AhR is widely expressed in the CNS (*Filbrandt* et al., 2004; *Jacob* et al., 2011). However, our understanding of the role of the AhR in neurons and supporting cells is still very limited. The BBB is vitally important in the maintenance of CNS homeostasis and its weakening has been suggested to contribute to neurodegenerative pathology. Breakdown of the BBB at the hippocampus has been correlated with cognitive impairment in humans (*Montagne* et al., 2015). Previously, our group reported that the BBB exhibits increased permeability in adult GF mice (*Braniste* et al., 2014). Monocolonization with *Clostridium tyrobutyricum* or *Bacteroides thetaiotaomicron* and treatment with sodium butyrate had rescuing effects on BBB permeability and tight junction protein expression (*Braniste* et al., 2014). One mechanism of the microbiome-mediated effects on BBB permeability appeared to be related to changes in the expression of tight junction proteins, such as occludin and claudin-5 (*Braniste* et al., 2014). A recent report demonstrated that induction of dysbiosis with a mixture of antibiotics caused alterations in the mRNA expression of tight junction proteins in the brain (*Frohlich* et al., 2016), validating, at an mRNA level, the results produced by *Braniste* et al. (2014) in a separate model of microbiome disruption.

The presence of the AhR and expression of its target genes has been shown to be significantly elevated in the micro-vessels of the brain (*Filbrandt* et al., 2004; *Dauchy* et al., 2008). Contradictory results have been reported. Via activation by TCDD, the AhR decreases the permeability of the BBB *in vivo* (*Wang* et al., 2011a,b), but increased BBB permeability was observed following exposure to 3-methylcholanthrene (*Chang* et al., 2012). Interestingly, though the increased BBB permeability reported by *Braniste* et al. (2014) has not been assessed in the context of the AhR, a recent study in keratinocytes demonstrated that ligand activation of the AhR elevates occludin and claudin 1 and 4 (*Takei* et al., 2015), indicating that a similar AhR-mediated effect could occur in the BBB. One of the most abundant gap junction proteins in the BBB is connexin 43. Connexin 43 expression and gap junction integrity has been shown to be down-regulated by AhR activation (*Andrysik* et al., 2013; *Kabatkova* et al., 2014). The deletion of connexin 43 is known to weaken the BBB, allowing it to open under increased vascular hydrostatic pressure or shear stress (*Ezan* et al., 2012). A recent report suggested that connexin 43 is integral to brain immune quiescence (*Boulay* et al., 2015) and, irrespective of BBB integrity, the deletion of connexin 43 was associated with increased immune cell recruitment across the BBB. Moreover, deletion of connexin 43 leads to activation of the endothelium and chemoattraction, thereby linking a key molecule in the maintenance of BBB integrity with the neuroinflammatory response (*Boulay* et al., 2015).

THE HOLOBIONT AND FURTHER DIRECTIONS

As mounting evidence supports the holobiont model of the host and its microbiome, one of the most important questions facing researchers are the mechanisms by which the microbiota communicates with the host. In humans, we have about 26,600 protein-encoding transcripts which are far fewer in number than that of the rice genome – approximately 46,000 functional transcripts. It is estimated that one thousand different strains of bacteria are expected to contribute up to $4x10^6$ potential mRNAs to the human transcriptome, thus making the human host-plus-microbiome genetic complexity closer to 4,026,600 mRNA transcripts. Thus, considering the holobiont concept, the complexity in humans outnumbers that of rice and other species. If we also bring in all the microRNAs etc., then the complexity of the human physiological road map becomes almost incomprehensible to take in. In conclusion, we have used the microbiome-AhR communication axis to illustrate a small glimpse of the considerable dynamics of microbiome host interactions. The AhR is an evolutionarily conserved ligand induced receptor involved in host-environment interactions. Despite its well-known responsiveness to man-made compounds, such as TCDD, the AhR does not elicit a response to dioxin in invertebrates. Therefore, AhR must execute other evolutionarily important roles in development and homeostasis and studies addressing this signalling pathway holds great promise for future understanding of the holobiont.

ACKNOWLEDGEMENT

This work is supported by grants from Swedish Medical Research Council, Singapore LKC School of Medicine and SCELSE NTU University.

LITERATURE

Andrysik, Z., Prochazkova, J., Kabatkova, M., Umannova, L., Simeckova, P., Kohoutek, J., Kozubik, A., Machala, M., and Vondracek, J.: Aryl hydrocarbon receptor-mediated disruption of contact inhibition is associated with connexin43 downregulation and inhibition of gap junctional intercellular communication. Arch. Toxicol. 87, 491-503 (2013).

Backhed, F., Manchester, J.K., Semenkovich, C.F., and Gordon, J.I.: Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc. Natl. Acad. Sci. USA 104, 979-984 (2007).

Badawy, A.A.: Tryptophan availability for kynurenine pathway metabolism across the life span: Control mechanisms and focus on aging, exercise, diet and nutritional supplements. Neuropharmacology 112, 248-263 (2015).

Badman, M.K., Pissios, P., Kennedy, A.R., Koukos, G., Flier, J.S., and Maratos-Flier, E.: Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metab. 5, 426-437 (2007).

Bell, E.A., Boehnke, P., Harrison, T.M., and Mao, W.L.: Potentially biogenic carbon preserved in a 4.1 billion-year-old zircon. Proc. Natl. Acad. Sci. USA 112, 14518-14521 (2015).

Benedict, J.C., Lin, T.M., Loeffler, I.K., Peterson, R.E., and Flaws, J.A.: Physiological role of the aryl hydrocarbon receptor in

mouse ovary development. Toxicol. Sci. 56, 382-388 (2000).

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., Macri, J., McCoy, K.D., Verdu, E.F., and Collins, S.M.: The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141, 599-609 (2011).

Berstad, A., Raa, J., and Valeur, J.: Tryptophan: 'essential' for the pathogenesis of irritable bowel syndrome? Scand. J. Gastroenterol. 49, 1493-1498 (2014).

Boulay, A.C., Mazeraud, A., Cisternino, S., Saubamea, B., Mailly, P., Jourdren, L., Blugeon, C., Mignon, V., Smirnova, M,. Cavallo, A., Ezan, P., Avé, P., Dingli, F., Loew, D., Vieira, P., Chrétien, F., and Cohen-Salmon, M.: Immune quiescence of the brain is set by astroglial connexin 43. J. Neurosci. 35, 4427-4439 (2015).

Braak, H., de Vos, R.A., Bohl, J., and Del Tredici, K.: Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neurosci. Lett. 396, 67-72 (2006).

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Toth, M., Korecka, A., Bakocevic, N., Guan, N.L., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B.T., Diamond, B., and Pettersson, S.: The gut microbiota influences blood-brain barrier permeability in mice. Sci. Transl. Med. 6, 263ra158 (2014).

Bull, G., Shattock, P., Whiteley, P., Anderson, R., Groundwater, P.W., Lough, J.W., and Lees, G.: Indolyl-3-acryloylglycine (IAG) is a putative diagnostic urinary marker for autism spectrum disorders. Med. Sci. Monit. 9, CR422-425 (2003).

Chang, C.C., Lee, P.S., Chou, Y., Hwang, L.L., and Juan, S.H.: Mediating effects of aryl-hydrocarbon receptor and RhoA in altering brain vascular integrity: the therapeutic potential of statins. Am. J. Pathol. 181, 211-221 (2012).

Chen, C.H., Lin, C.L., and Kao, C.H.: Irritable Bowel Syndrome Is Associated with an Increased Risk of Dementia: A Nationwide Population-Based Study. PloS One 11, e0144589 (2016).

Cheng, X., Vispute, S.G., Liu, J., Cheng, C., Kharitonenkov, A., and Klaassen, C.D.: Fibroblast growth factor (Fgf) 21 is a novel target gene of the aryl hydrocarbon receptor (AhR). Toxicol. Appl. Pharmacol. 278, 65-71 (2014).

Chng, S.H., Kundu, P., Dominguez-Brauer, C., Teo, W.L., Kawajiri, K., Fujii-Kuriyama, Y., Mak, T.W., and Pettersson, S.: Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity. Sci. Rep. 6, 23820 (2016).

Coskun, T., Bina, H.A., Schneider, M.A., Dunbar, J.D., Hu, C.C., Chen, Y., Moller, D.E., and Kharitonenkov, A.: Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 149, 6018-6027 (2008).

Dauchy, S., Dutheil, F., Weaver, R.J., Chassoux, F., Daumas-Duport, C., Couraud, P.O., Scherrmann, J.M., De Waziers, I., and Decleves, X.: ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. J. Neurochem. 107, 1518-1528 (2008).

Denison, M.S. and Nagy, S.R.: Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309-334 (2003).

Diani-Moore, S., Ram, P., Li, X., Mondal, P., Youn, D.Y., Sauve, A.A., and Rifkind, A.B.: Identification of the aryl hydrocarbon receptor target gene TiPARP as a mediator of suppression of hepatic gluconeogenesis by 2,3,7,8-tetrachlorodibenzo-p-dioxin and of nicotinamide as a corrective agent for this effect. J. Biol. Chem. 285, 38801-38810 (2010).

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., and Pettersson, S.: Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad.

Sci. USA 108, 3047-3052 (2011).
DiNatale, B.C., Murray, I.A., Schroeder, J.C., Flaveny, C.A., Lahoti, T.S., Laurenzana, E.M., Omiecinski, C.J., and Perdew, G.H.: Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling. Toxicol. Sci. 115, 89-97 (2010).
Duarte, J.H., Di Meglio, P., Hirota, K., Ahlfors, H., and Stockinger, B.: Differential influences of the aryl hydrocarbon receptor on Th17 mediated responses in vitro and in vivo. PloS One 8, e79819 (2013).
Ezan, P., Andre, P., Cisternino, S., Saubamea, B., Boulay, A.C., Doutremer, S., Thomas, M.A., Quenech'du, N., Giaume, C., and Cohen-Salmon, M.: Deletion of astroglial connexins weakens the blood-brain barrier. J. Cereb. Blood Flow Metab. 32, 1457-1467 (2012).
Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M., and Gonzalez, F.J.: Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 268, 722-726 (1995).
Filbrandt, C.R., Wu, Z., Zlokovic, B., Opanashuk, L., and Gasiewicz, T.A.: Presence and functional activity of the aryl hydrocarbon receptor in isolated murine cerebral vascular endothelial cells and astrocytes. Neurotoxicology 25, 605-616 (2004).
Frohlich, E.E., Farzi, A., Mayerhofer, R., Reichmann, F., Jacan, A., Wagner, B., Zinser, E., Bordag, N., Magnes, C., Frohlich, E., Kashofer, K., Gorkiewicz, G., and Holzer, P.: Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. Brain Behav. Immun. 56, 140-155 (2016).
Gandhi, R., Kumar, D., Burns, E.J., Nadeau, M., Dake, B., Laroni, A., Kozoriz, D., Weiner, H.L., and Quintana, F.J.: Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells. Nature Immunol. 11, 846-853 (2010).
Gohlke, J.M., Stockton, P.S., Sieber, S., Foley, J., and Portier, C.J.: AhR-mediated gene expression in the developing mouse telencephalon. Reprod. Toxicol. 28, 321-328 (2009).
Hahn, M.E., Karchner, S.I., Shapiro, M.A., and Perera, S.A.: Molecular evolution of two vertebrate aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. Proc. Natl. Acad. Sci. USA 94, 13743-13748 (1997).
Hahn, M.E.: Aryl hydrocarbon receptors: diversity and evolution. Chem. Biol. Interact. 141, 131-160 (2002).
Hao, N. and Whitelaw, M.L.: The emerging roles of AhR in physiology and immunity. Biochem. Pharmacol. 86, 561-570 (2013).
Harach, T., Marungruang, N., Dutilleul, N., Cheatham, V., McCoy, K.D., Neher, J.J., Jucker, M., Fak, F., Lasser, T., and Bolmont, T.: Reduction of Alzheimer's disease beta-amyloid pathology in the absence of gut microbiota. arXiv:1509.02273 [q-bioMN] (2015).
Hill, A., Howard, C.V., Strahle, U., and Cossins, A.: Neurodevelopmental defects in zebrafish (Danio rerio) at environmentally relevant dioxin (TCDD) concentrations. Toxicol. Sci. 76, 392-399 (2003).
Hoban, A.E., Stilling, R.M., Ryan, F.J., Shanahan, F., Dinan, T.G., Claesson, M.J., Clarke, G., and Cryan, J.F.: Regulation of prefrontal cortex myelination by the microbiota. Transl. Psychiatry 6, e774 (2016).
Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F., Patteerson, P.H., and Mazmanian, S.K.: Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155, 1451-1463 (2013).
Hsu, P., Lee, P.Y., and Lee, Y.H.: The role of Aryl Hydrocarbon Receptor in the excitatory synaptic protein expression in cortical neurons and brain inflammation. 9th FENS Forum of Neuroscience, Milan, Italy, (2014).
Hu, W., Sorrentino, C., Denison, M.S., Kolaja,

K., and Fielden, M.R.: Induction of cyp1a1 is a nonspecific biomarker of aryl hydrocarbon receptor activation: results of large scale screening of pharmaceuticals and toxicants in vivo and in vitro. Mol. pharmacol. 71, 1475-1486 (2007).

Huang, X., Powell-Coffman, J.A., and Jin, Y.: The AHR-1 aryl hydrocarbon receptor and its co-factor the AHA-1 aryl hydrocarbon receptor nuclear translocator specify GABAergic neuron cell fate in C. elegans. Development 131, 819-828 (2004).

Hubbard, T.D., Murray, I.A., Bisson, W.H., Lahoti, T.S., Gowda, K., Amin. S.G., Patterson, A.D., and Perdew, G.H.: Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. Sci. Rep. 5, 12689 (2015).

Inagaki, T., Dutchak, P., Zhao, G., Ding, X., Gautron, L., Parameswara, V., Li, Y., Goetz, R., Mohammadi, M., Esser, V., Elmquist, J.K., Gerard, R.D., Burgess, S.C., Hammer, R.E., Mangelsdorf, D.J., and Kliewer, S.A.: Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab. 5, 415-425 (2007).

Jacob, A., Hartz, A.M., Potin, S., Coumoul, X., Yousif, S., Scherrmann, J.M., Bauer, B., and Decleves, X.: Aryl hydrocarbon receptor-dependent upregulation of Cyp1b1 by TCDD and diesel exhaust particles in rat brain microvessels. Fluids Barriers CNS 8, 23 (2011).

Jin, G.B., Winans, B., Martin, K.C., and Lawrence, B.P.: New insights into the role of the aryl hydrocarbon receptor in the function of CD11c(+) cells during respiratory viral infection. Eur. J. Immunol. 44, 1685-1698 (2014a).

Jin, U.H., Lee, S.O., Sridharan, G., Lee, K., Davidson, L.A., Jayaraman, A., Chapkin, R.S., Alaniz, R., and Safe, S.: Microbiome-derived tryptophan metabolites and their aryl hydrocarbon receptor-dependent agonist and antagonist activities. Mol. Pharmacol. 85, 777-788 (2014b).

Kabatkova, M., Svobodova, J., Pencikova, K., Mohatad, D.S., Smerdova, L., Kozubik, A., Machala, M., and Vondracek, J.: Interactive effects of inflammatory cytokine and abundant low-molecular-weight PAHs on inhibition of gap junctional intercellular communication, disruption of cell proliferation control, and the AhR-dependent transcription. Toxicol. Lett. 232, 113-121 (2014).

Kelly, L.P., Carvey, P.M., Keshavarzian, A., Shannon, K.M., Shaikh, M., Bakay, R.A., and Kordower, J.H.: Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. Mov. Disord. 29, 999-1009 (2014).

Kharitonenkov, A., Shiyanova, T.L., Koester, A., Ford, A.M., Micanovic, R., Galbreath, E.J., Sandusky, G.E., Hammond, L.J., Moyers, J.S., Owens, R.A., Gromada, J., Brozinick, J.T., Hawkins, E.D., Wroblewski, V.J., Li, D.-E., Mehrbod, F., Jaskunas, S.R., and Shanafelt, A.B.: FGF-21 as a novel metabolic regulator. J. Clin. Invest. 115, 1627-1635 (2005).

Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T.: Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. J. Exp. Med. 206, 2027-2035 (2009).

Kimura, E., Ding, Y., and Tohyama, C.: AhR signaling activation disrupts migration and dendritic growth of olfactory interneurons in the developing mouse. Sci. Rep. 6, 26386 (2016).

Korecka, A., Dona, A., Lahiri, S.K., Tett, A.J., Al-Asmakh, M., Braniste, V., D'Arienzo, R., Abbaspour, A., Reichardt, N., Fujii-Kuriyama, Y., Rafter, J., Narbad, A., Holmes, E., Nicholson, J., Arulampalam, V., and Pettersson, S.: Bidirectional communication between the Aryl-hydrocarbon-Receptor (AhR) and the gut microbiome tunes host metabolism. NPJ Biofilms Microbiomes 2, pii: 16014 (2016).

Krajmalnik-Brown, R., Lozupone, C., Kang, D.W., and Adams, J.B.: Gut bacteria in children with autism spectrum disorders: challenges and promise of studying how a

complex community influences a complex disease. Microb. Ecol. Health Dis. 26, 26914 (2015).

Lai, S.W., Liao, K.F., Lin, C.L., and Sung, F.C.: Irritable bowel syndrome correlates with increased risk of Parkinson's disease in Taiwan. Eur. J. Epidemiol. 29, 57-62 (2014).

Latchney, S.E., Hein, A.M., O'Banion, M.K., DiCicco-Bloom, E., and Opanashuk, L.A.: Deletion or activation of the aryl hydrocarbon receptor alters adult hippocampal neurogenesis and contextual fear memory. J. Neurochem. 125, 430-445 (2013).

Lee, J.H., Wada, T., Febbraio, M., He, J., Matsubara, T., Lee, M.J., Gonzalez, F.J., and Xie, W.: A novel role for the dioxin receptor in fatty acid metabolism and hepatic steatosis. Gastroenterology 139, 653-663 (2010).

Linden, J., Lensu, S., Tuomisto, J., and Pohjanvirta, R.: Dioxins, the aryl hydrocarbon receptor and the central regulation of energy balance. Front. Neuroendocrinol. 31, 452-478 (2010).

Lu, H., Cui, W., and Klaassen, C.D.: Nrf2 protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced oxidative injury and steatohepatitis. Toxicol. Appl. Pharmacol. 256, 122-135 (2011).

Lu, P., Yan, J., Liu, K., Garbacz, W.G., Wang, P., Xu, M., Ma, X., and Xie, W.: Activation of aryl hydrocarbon receptor dissociates fatty liver from insulin resistance by inducing fibroblast growth factor 21. Hepatology 61, 1908-1919 (2015).

Luczynski, P., Whelan, S.O., O'Sullivan, C., Clarke, G., Shanahan, F., Dinan, T.G., and Cryan, J.F.: Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus. Eur. J. Neurosci. 44, 2654-2666 (2016).

Ma, Q., Baldwin, K.T., Renzelli, A.J., McDaniel, A., and Dong, L.: TCDD-inducible poly(ADP-ribose) polymerase: a novel response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biochem. Biophys. Res. Commun. 289, 499-506 (2001).

Maloof, A.C., Rose, C.V., Beach, R., Samuels, B.M., Calmet, C.C., Erwin, D.H., Poirier, G.R., Yao, N., and Simons, F.J.: Possible animal-body fossils in pre-Marinoan limestones from South Australia. Nat. Geosci. 3, 653-659 (2010).

McPherson, Z.E., Talley, N.J., Walker, M., McElduff, P., Attia, J., Kelly, B., Agar, A., Coroneo, M.T., Petterson, S., Sorensen, H., White, A.J., and McEvoy, M.:. Su1088A Novel Predictive Association Between Irritable Bowel Syndrome and Glaucomatous Optic Neuropathy. Gastroenterology 148, Suppl. 1, S-404 (2015).

Mezrich, J.D., Fechner, J.H., Zhang, X., Johnson, B.P., Burlingham, W.J., and Bradfield, C.A.: An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J. Immunol. 185, 3190-3198 (2010).

Möhle, L., Mattei, D., Heimesaat, M.M., Bereswill, S., Fischer, A., Alutis, M., French, T., Hambardzumyan, D., Matzinger, P., Dunay, I.R., and Wolf, S.A.: Ly6C(hi) Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. Cell Rep. 15, 1945-1956 (2016).

Montagne, A., Barnes, S.R., Sweeney, M.D., Halliday, M.R., Sagare, A.P., Zhao, Z., Toga, A.W., Jacobs, R.E., Liu, C.Y., Amezcua, L., Harrington, M.G., Chui, H.C., Law, M., and Zlokovic, B.V.: Blood-brain barrier breakdown in the aging human hippocampus. Neuron 85, 296-302 (2015).

Montagner, A., Korecka, A., Polizzi, A., Lippi, Y., Blum, Y., Canlet, C., Tremblay-Franco, M., Gautier-Stein, A., Burcelin, R., Yen, Y.-C., Je, H.S., Maha, A.-A., Mithieux, G., Arulampalam, V., Lagarrigue, S., Guillou, H., Pettersson, S., and Wahlic, W.: Hepatic circadian clock oscillators and nuclear receptors integrate microbiome-derived signals. Sci. Rep. 6, 20127 (2016).

Moura-Alves, P., Fae, K., Houthuys, E., Dorhoi, A., Kreuchwig, A., Furkert, J., Barison, N., Diehl, A., Munder, A., Constant, P., Skrahina, T., Guhlich-Bornhof, U., Klemm, M., Koehler, A.B., Bandermann,

S., Goosmann, C., Mollenkopf, H.J., Hurwitz, R., Brinkmann, V., Fillatreau, S., Daffe, M., Tümmler, B., Kolbe, M., Oschkinat, H., Krause, G., and Kaufmann, S.H.: AhR sensing of bacterial pigments regulates antibacterial defence. Nature 512, 387-392 (2014).

Murray, I.A., Patterson, A.D., and Perdew, G.H.: Aryl hydrocarbon receptor ligands in cancer: friend and foe. Nat. Rev. Cancer 14, 801-814 (2014).

Nguyen, L.P. and Bradfield, C.A.: The search for endogenous activators of the aryl hydrocarbon receptor. Chem. Res. Toxicol. 21, 102-116 (2008).

Nguyen, N.T., Hanieh, H., Nakahama, T., and Kishimoto, T.: The roles of aryl hydrocarbon receptor in immune responses. Int. Immunol. 25, 335-343 (2013).

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., and Pettersson, S.: Host-gut microbiota metabolic interactions. Science 336, 1262-1267 (2012).

Ogbonnaya, E.S., Clarke, G., Shanahan, F., Dinan, T.G., Cryan, J.F., and O'Leary, O.F.: Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. Biol. Psychiatry 78, e7-9 (2015).

Opitz, C.A., Litzenburger, U.M., Sahm, F., Ott, M., Tritschler, I., Trump, S., Schumacher, T., Jestaedt, L., Schrenk, D., Weller, M., Jugold, M., Guillemin, G.J., Miller, C.L., Lutz, C., Radlwimmer, B., Lehmann, I., von Deimling, A., Wick, W., and Platten, M.: An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 478, 197-203 (2011).

Pocivavsek, A., Wu, H.Q., Elmer, G.I., Bruno, J.P., and Schwarcz, R.: Pre- and postnatal exposure to kynurenine causes cognitive deficits in adulthood. Eur. J. Neurosci. 35, 1605-1612 (2012).

Qin, H. and Powell-Coffman, J.A.: The Caenorhabditis elegans aryl hydrocarbon receptor, AHR-1, regulates neuronal development. Dev. Biol. 270, 64-75 (2004).

Qin, H., Zhai, Z., and Powell-Coffman, J.A.: The Caenorhabditis elegans AHR-1 transcription complex controls expression of soluble guanylate cyclase genes in the URX neurons and regulates aggregation behavior. Dev. Biol. 298, 606-615 (2006).

Quintana, F.J., Basso, A.S., Iglesias, A.H., Korn, T., Farez, M.F., Bettelli, E., Caccamo, M., Oukka, M., Weiner, H.L.: Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature 453, 65-71 (2008).

Quintana, F.J., Murugaiyan, G., Farez, M.F., Mitsdoerffer, M., Tukpah, A.M., Burns, E.J., and Weiner, H.L.: An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. USA 107, 20768-20773 (2010).

Rabot, S., Membrez, M., Bruneau, A., Gerard, P., Harach, T., Moser, M., Raymond, F., Mansourian, R., and Chou, C.J.: Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. FASEB J. 24, 4948-4959 (2010).

Remillard, R.B. and Bunce, N.J.: Linking dioxins to diabetes: epidemiology and biologic plausibility. Environ. Health Perspect. 110, 853-858 (2002).

Rosenfeld, C.S.: Microbiome Disturbances and Autism Spectrum Disorders. Drug Metab. Dispos. 43, 1557-1571 (2015).

Scheperjans, F., Aho, V., Pereira, P.A., Koskinen, K., Paulin, L., Pekkonen, E., Haapaniemi, E., Kaakkola, S., Eerola-Rautio, J., Pohja, M., Kinnunen, E., Murros, K., and Auvinen, P.: Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov. Disord. 30, 350-358 (2015).

Shertzer, H.G. and Senft, A.P.: The micronutrient indole-3-carbinol: implications for disease and chemoprevention. Drug Metabol. Drug Interact. 17, 159-188 (2000).

Stockinger, B., Di Meglio, P., Gialitakis, M., and Duarte, J.H.: The aryl hydrocarbon receptor: multitasking in the immune system. Annu. Rev. Immunol. 32, 403-432 (2014).

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C., and Koga, Y.: Postnatal microbial colonization pro-

grams the hypothalamic-pituitary-adrenal system for stress response in mice. J. Physiol. 558, 263-275 (2004).

Takei, K., Mitoma, C., Hashimoto-Hachiya, A., Takahara, M., Tsuji, G., Nakahara, T., and Furue, M.: Galactomyces fermentation filtrate prevents T helper 2-mediated reduction of filaggrin in an aryl hydrocarbon receptor-dependent manner. Clin. Exp. Dermatol. 40, 786-793 (2015).

Tran, N.N., Pham, T.T., Ozawa, K., Nishijo, M., Nguyen, A.T., Tran, T.Q., Hoang, L.V., Tran, A.H., Phan, V.H., Nakai, A., Nishino, Y., and Nishijo, H.:. Impacts of Perinatal Dioxin Exposure on Motor Coordination and Higher Cognitive Development in Vietnamese Preschool Children: A Five-Year Follow-Up. PloS One 11, e0147655 (2016).

Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier, L., Renauld, J.C., and Stockinger, B.: The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature 453, 106-109 (2008).

Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A., and Stockinger, B.: Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. J. Exp. Med. 206, 43-49 (2009).

Vorderstrasse, B.A., Bohn, A.A., and Lawrence, B.P.: Examining the relationship between impaired host resistance and altered immune function in mice treated with TCDD. Toxicology 188, 15-28 (2003).

Wang, X., Hawkins, B.T., and Miller, D.S.: Activating PKC-beta1 at the blood-brain barrier reverses induction of P-glycoprotein activity by dioxin and restores drug delivery to the CNS. J. Cereb. Blood Flow Metab. 31, 1371-1375 (2011a).

Wang, X., Hawkins, B.T., and Miller, D.S.: Aryl hydrocarbon receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood-brain barrier. FASEB J. 25, 644-652 (2011b).

Warren, T.K., Mitchell, K.A., and Lawrence, B.P.: Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suppresses the humoral and cell-mediated immune responses to influenza A virus without affecting cytolytic activity in the lung. Toxicol. Sci. 56, 114-123 (2000).

Williamson, M.A., Gasiewicz, T.A., and Opanashuk, L.A.: Aryl hydrocarbon receptor expression and activity in cerebellar granule neuroblasts: implications for development and dioxin neurotoxicity. Toxicol. Sci. 83, 340-348 (2005).

Xu, C.X., Wang, C., Zhang, Z.M., Jaeger, C.D., Krager, S.L., Bottum, K.M., Liu, J., Liao, D.F., and Tischkau, S.A.: Aryl hydrocarbon receptor deficiency protects mice from diet-induced adiposity and metabolic disorders through increased energy expenditure. Int. J. Obes. (Lond.) 39, 1300-1309 (2015).

Yamada, T., Horimoto, H., Kameyama, T., Hayakawa, S., Yamato, H., Dazai, M., Takada, A., Kida, H., Bott, D., Zhou, A.C., Hutin, D., Watts, T.H., Asaka, M., Matthews, J., and Takaoka, A.: Constitutive aryl hydrocarbon receptor signaling constrains type I interferon-mediated antiviral innate defense. Nature Immunol. 17, 687-694 (2016).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 93-113 (2017).

SYMBIOTIC BACTERIA AND THEIR THERAPEUTIC POTENTIAL

VOLKER RUSCH[1,2], TRUDY M. WASSENAAR[3],
and KURT ZIMMERMANN[4]

[1]Old Herborn University Foundation, Herborn, Germany
[2]Institute for Integrative Biology, Herborn, Germany
[3]Molecular Microbiology and Genomics Consultants, Zotzenheim, Germany
[4]SymbioPharm GmbH, Herborn, Germany

INTRODUCTION

The majority of probiotic products currently on the market for human use are based on Lactic Acid bacteria (Lactobacillales), and many contain species of the genera *Lactobacillus* or *Lactococcus* (members of the phylum of Firmicutes) or *Bifidobacteria* (which were reclassified from '*Lactobacillus bifidum*' to a genus within the Actinobacteria phylum). What these bacteria have in common is their production of lactic acid as a result of carbohydrate fermentation. They are also frequently used as starter cultures for dairy products and other fermented foods.

Other, less frequently encountered probiotic bacterial species are apathogenic strains of *Enterococcus faecalis* (another Lactobacillales) and *Escherichia coli* (a Gamma-proteobacterium). On the market are products containing live *Enterococcus faecalis*, live *Escherichia coli*, and a bacterial lysate of *Enterococcus faecalis* and *Escherichia coli* (see chapter: "Identification of bacterial products" described later in this publication).

ENTEROCOCCUS FAECALIS PREPARATION

Animal studies with *Enterococcus faecalis* SY

Originally, the product based on apathogenic *E. faecalis* was described as 'oral vaccine' against enteropathogens such as *Salmonella*. Results obtained with mouse experiments were summarized in a publication that demonstrated some protection against *S. typhimurium* challenge and protection against intraperitoneal challenge with *Haemophilus influenzae* was also shown (*Rusch* et al., 1983). The presumed protection by probiotic bacteria against infections had already been proposed by Alfred Nissle, who explained the observed effect by *in situ* competition (*Nissle*, 1916). Instead, Rusch and co-workers preferred the hypothesis that the effect was the result of stimulation of 'non-specific immune activities'. This hypothesis was investigated in a follow up murine study that used *E. faecalis* SY (*Hyde* et al., 1986). Mice received the *E. faecalis* bacteria (still called *Streptococcus faecalis* in those days) via their drinking water for 3 weeks, after which the animals were sacrificed to isolate polymorphonuclear leukocytes (PMNs), macrophages and Natural Killer (NK) cells. The PMNs of treated animals were able to kill *Staphylococcus aureus* bacteria more efficiently than PMNs from untreated mice. However, neither the intracellular killing of *S. typhimurium* in macro-

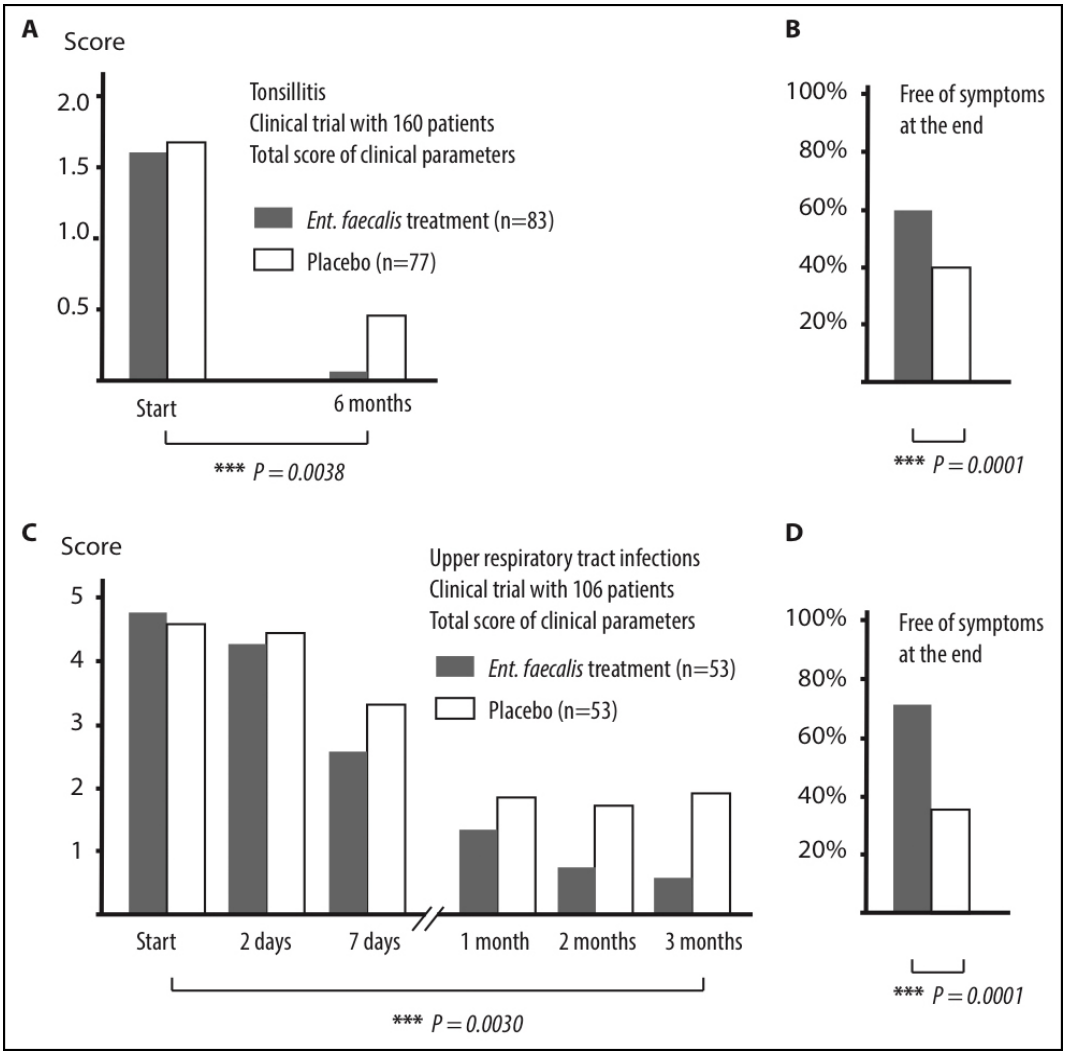

Figure 1: Results from two clinical trials with *Enterococcus faecalis* SY.
Panels A and B summarize findings for a trial with 160 patients suffering from chronic tonsillitis, Panels C and D results from the trial with 106 patients with frequent upper respiratory tract infections. Shown are total clinical scores (Panels A and C), and the percentage of patients free of symptoms at the end (Panels Band D). (Modified after *Rusch* et al., 1986; *Kalinski*, 1986, 1987).

phages, nor the activity of Natural Killer (NK) cells was enhanced as a result of the treatment (*Hyde* et al., 1986).

An animal study using 8-12 months-old pigs was used to investigate the immune modulation of *E. faecalis* SY in more detail. Animals were given various doses of the product to determine that twice a daily dose of 10^9 bacteria per animal was required to produce increased cytotoxicity of peripheral granulocytes (*Ottendorfer* et al., 1995). Gut-associated lymphoid tissue (GALT) was isolated *post mortem* to assess local, short-term effects of presence of these probiotic bacteria. The PHA stimulation of lymphocytes isolated from the mesenteric lymph nodes was increased in exposed animals, while the nodes also contained more IgA-producing cells. Moreover, the s-IgA

levels in saliva were elevated (*Ottendorfer* et al., 1995). All these effects were considered to be in line with a mechanistic immune-modulation by *E. faecalis* SY that could explain its positive effects on human conditions such as COPD and recurrent airway infections.

Clinical trials with *Enterococcus faecalis*

Two double blind placebo-controlled clinical trials were summarized in an early publication from 1986, one describing 160 patients with chronic tonsillitis and the other involving 106 patients suffering from various respiratory tract infections (*Rusch* et al., 1986). In both trials, the patients were treated with *E. faecalis* SY or placebo (the tonsillitis trial treatment lasted 6 months, in the other trial patients were treated for 3 months). In both trials, the immunological outcome was determined by the Merieux Multitest, a multi-antigen skin test that was frequently used in those days, and by assessment of the patient's health status by a physician. Further, immunoglobulin titres were determined in saliva and serum. At the end of the tonsillitis trial, which is described in more detail elsewhere (*Kalinksi*, 1986), both patient groups had improved their total scores for clinical parameters, but the group receiving treatment had significantly better scores ($p = 0.0038$) compared to the placebo group. Moreover, 65% of the patients receiving the probiotic were free of symptoms at the end of treatment, compared to 35% of the placebo group ($p=0.0001$). The key data from this trial are summarized in Figure 1 panels A and B.

The other trial targeted patients with 'impaired immune conditions' as judged by the frequency of chronic upper respiratory tract infections (*Kalinksi*, 1987). Again the difference in total clinical scores was highly significant between treatment and placebo group ($p=0.0003$), and in this trial the Merieux Multitest resulted in significantly higher reactivity in the treatment group ($p=0.0280$), while serum levels of IgA and IgM significantly increased compared to placebo. In the verum group 40 patients became free of symptoms (61%) compared to 20 patients in the placebo group (38%, $p=0.001$). This is summarized in Figure 1 panels C and D. From these two trials it was concluded that *E. faecalis* SY provided an effective and safe treatment for chronic upper respiratory tract infections due to immunodeficiencies (*Rusch* et al., 1986).

The level of circulating antibodies was subject of a study by Jansen and co-workers, who determined humoral IgG levels directed against *E. faecalis* in 10 healthy volunteers who daily took 10^7 bacteria of *E. faecalis* SY for 3 weeks (*Jansen* et al., 1993). Their post-intake serum IgG levels were reduced compared to two samples taken (5 weeks apart) prior to intake, and the reduction was even stronger 3 weeks after the last intake, while IgG levels returned back to their original values 6 months later. The decrease in circulating IgG levels was interpreted as the result of an anti-inflammatory effect by the product (*Jansen* et al., 1993).

Another double-blind, placebo-controlled clinical trial was performed to evaluate the effect of *E. faecalis* SY on chronic recurrent bronchitis (*Habermann* et al., 2001). A total of 136 patients were randomly divided over a placebo group of 66 and a treatment group of 70 individuals. The treatment or placebo intake lasted for 6 months, after which the groups were followed for another 8 months. The treatment group suffered from 39 bronchitis incidents, which was 60% less than the 66 cases occurring in the placebo group

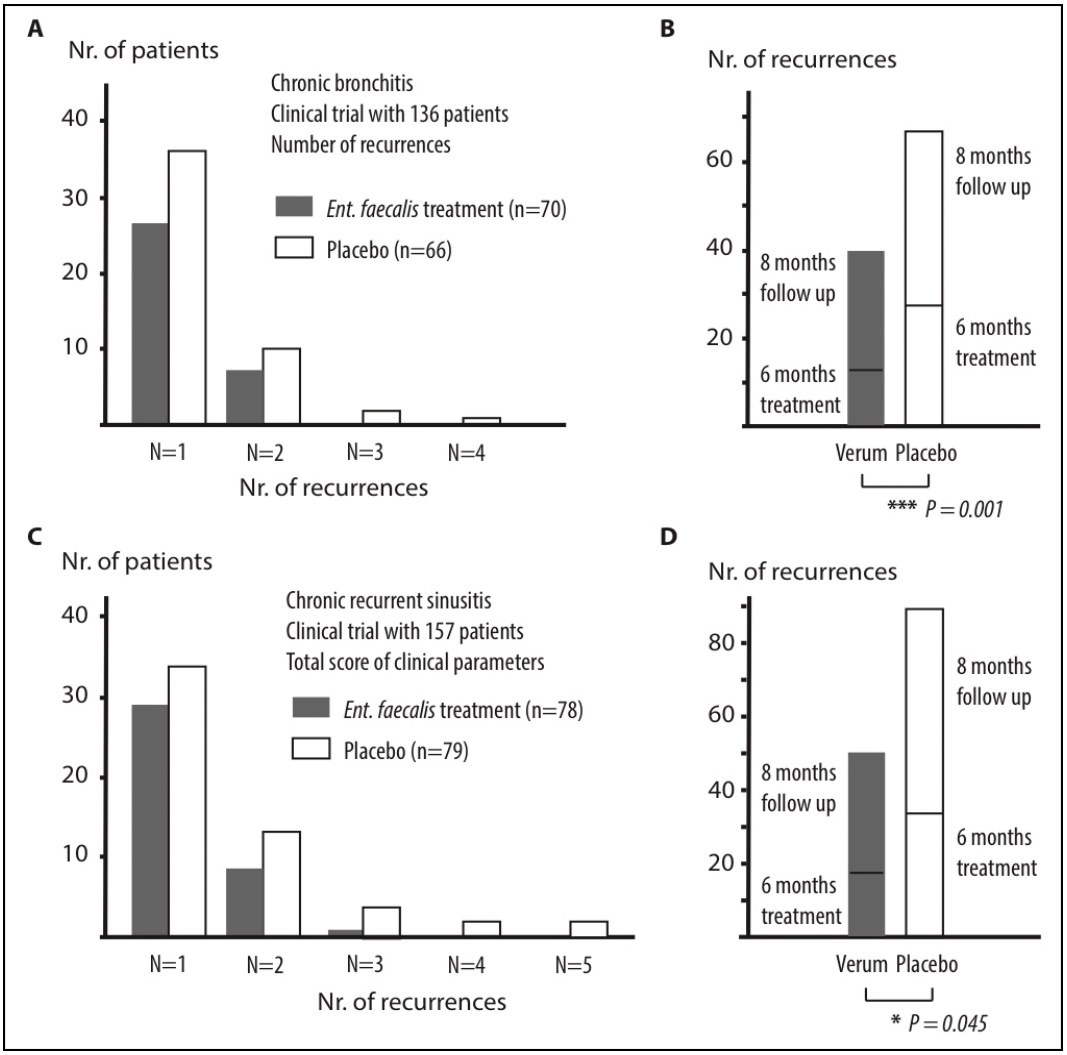

Figure 2: Results from two clinical trials with *Enterococcus faecalis* SY.
Panels A and B summarize findings for a trial with 136 patients suffering from chronic bronchitis. Panels C and D reports results from the trial with 157 patients with frequent sinusitis. Shown are the number of recurrences (Panels A and C), and the total number of recurrences during the 6 months of treatment and 8 months of follow-up (Panels B and D). The latter show P-values by Kaplan-Meier analysis. (Modified after *Habermann* et al., 2001, 2002).

(Figure 2, panels A and B). Treatment with the product further resulted in delayed relapse, fewer episodes, and less severe symptoms, compared to the placebo group. Side effects were not observed in either group (*Habermann* et al., 2001). Another publication from the same research group came out a year later, describing 157 patients suffering from chronic recurrent hypertrophic sinusitis (*Habermann* et al., 2002). The followed procedure was similar to the previous study, and this time 50 sinusitis incidents were observed in the treatment group of 78 patients, compared to 90 cases occurring in the placebo group of 79 patients, a significant difference (Figure 2 panels C and D). A difference in frequency of sinusitis was observed during treatment

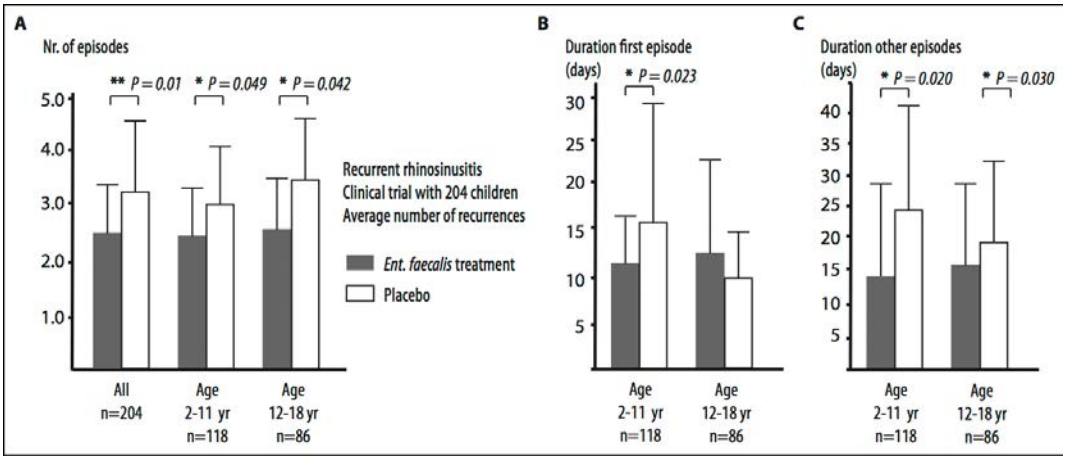

Figure 3: Results of a clinical trial with *Enterococcus faecalis* SY involving 204 children suffering from recurrent rhino-sinusitis.
Panel A reports the average number of episodes for all patients and for the two age groups that were separately analysed. Panel B shows the average duration of the first episode, and Panel C the average duration of other episodes, both for the two age groups. (Modified after *Kitz* et al., 2012).

as well as during the 8 months thereafter and, as with recurrent bronchitis, the time it took for the first relapse to occur was longer after treatment (*Habermann* et al., 2002).

Kitz and colleagues determined the efficacy of *E. faecalis* SY to treat recurrent sinusitis in children (*Kitz* et al., 2012). The study was a prospective trial, in which children suffering from recurrent rhino-sinusitis were assigned to standard treatment, with or without a subsequent 60-days course of the probiotic bacteria. Children in the treatment group had fewer episodes than the controls (p=0.01). The duration of the first episode was significantly shorter in the younger treatment group, and duration of other episodes was shorter for both age groups (Figure 3). A delay in onset of the first relapse and reduced severity of symptoms was marginally different between the two groups (*Kitz* et al., 2012).

A systematic review that addressed the application of probiotics for prevention of respiratory tract infections was published in 2009 (*Vouloumanou* et al., 2009). A total of 109 publications were identified in PubMed, of which 14 described randomised clinical trials (RCTs) that fulfilled all criteria for inclusion; this included the Habermann 2002 study. Most other included RCTs had tested *Lactobacillus* species, while two trials had used *Bifidobacterium* species. In contrast to the results reported by Habermann and colleagues, 10 of the 14 compared studies reported no difference in incidence of respiratory tract infections between treatment and placebo control group (*Vouloumanou* et al., 2009).

Mechanistic model of action of *E. faecalis* SY

The mechanism of action of *E. faecalis* SY can be summarized as schematically presented in Figure 4. The bacteria activate monocytes/macrophages, which react by production of interleukin-1β (IL-1β) and IL-6. This stimulates the activation and subsequent proliferation of dormant B cells, with the production of IgA and s-IgA as a consequence.

This model is based on several independent observations. That *E. faecalis* SY bacteria resulted in IL-1β and IL-6 release by human PMNs was demon-

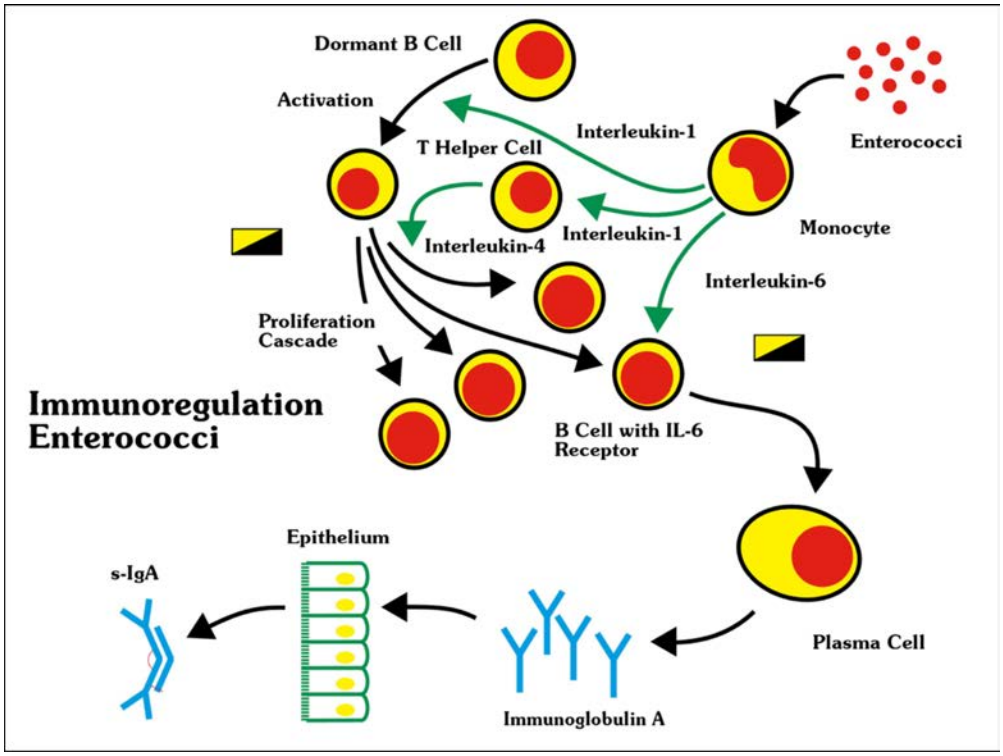

Figure 4: Model of immunoregulation by *Enterococcus faecalis* SY.
For explanation see text. (Taken from *Rusch* and *Zimmermann*, 1995).

strated *in vitro*; the cytokine production was dose-dependent and could be inhibited by dexamethasone but not by cyclosporine A (*Rosenkranz* and *Grundmann*, 1994). Production of interferon gamma (IFN-γ) was weaker but also dose-dependant, and could be inhibited by both dexamethasone and cyclosporine A. Proliferation of B cells was demonstrated in the pig model described above, while increased s-IgA levels in saliva were also demonstrated (*Ottendorfer* et al., 1995). The human clinical trials had produced data on an increase of serum IgA and IgM (*Rusch* et al., 1986; *Kalinksi*, 1986, 1987).

A random selection of the tonsillitis patients who had participated in the clinical trial previously mentioned (*Rusch* et al., 1986) had donated blood before the beginning of treatment, at 4 timepoints during treatment and again 3 months after treatment was finished. Monocytes isolated from these blood samples were tested for their production of IFN-γ, as well as IL-2, IL-1β and IL-6 (R. Kunze and H. Skarabis, unpublished data). As expected, the levels of these immune responses varied considerably between individuals, and some levels were too high or too low to be determined quantitatively by means of a standard curve. For those measures that were accurate, the trends prior to treatment and during treatment were compared for patients from the verum and treatment groups. Some of the obtained data are summarized in Figure 5. Notably, the production of IFN-γ in response to PHA+ *E. faecalis* SY stimulation was down-regulated in monocytes obtained from treated patients, compared to controls. This is shown in Panels 5A-C, illustrated by the decreased slope of the red curve (verum group) in panels B and C.

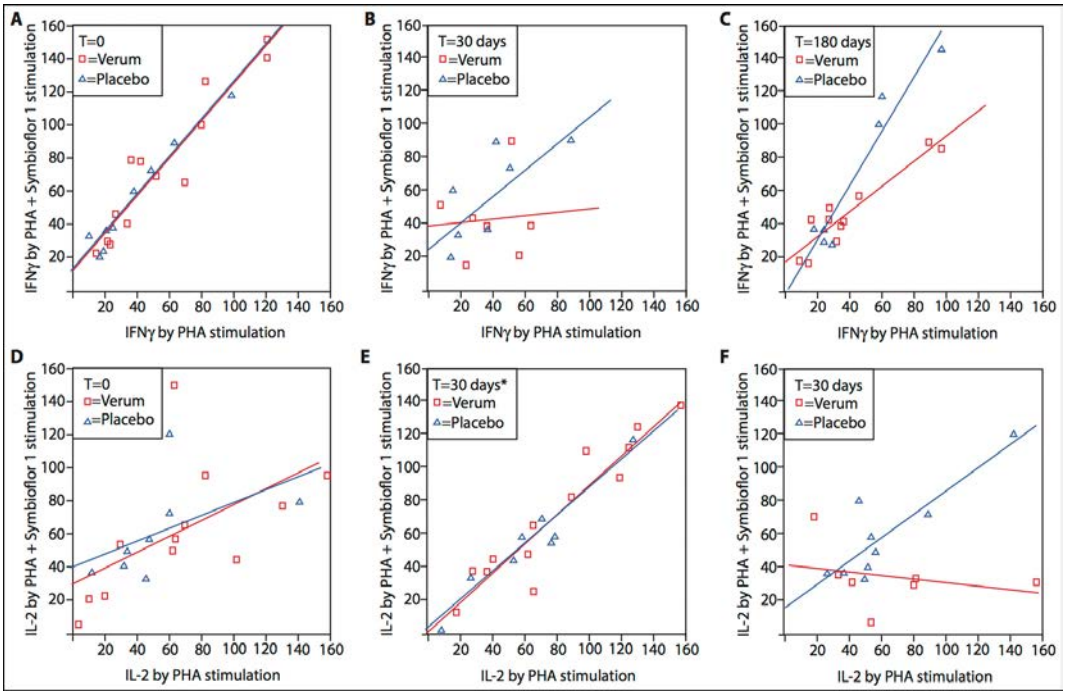

Figure 5: Production of immune regulators by monocytes isolated from patients who participated in the tonsillitis trial.
Panels A-C show the IFN-γ production after stimulation with PHA plotted against stimulation with PHA combined with *E. faecalis* SY. Panels D-F show the results for IL-2 production. Blood samples were taken prior to treatment (Panels A, D), after 30 days of treatment (Panels B, E, F) and after 180 days (panel C). Note that Panel E shows the outcome read after 15 hrs. of stimulation (marked by *), while D and F are read after 48 hrs. of stimulation. (Modified after R. Kunze and H. Skarabis, unpublished data).

When the levels of produced IL-2 were measured after 15 hrs. of incubation, they remained more or less the same over time, either in the verum or placebo group (data are shown for day 30 only, panel 5E, where the verum and treatment curve overlap), whereas the production levels of IL-2 differed significantly between the two treatment groups when read after 48 hr. This is shown in Panel 5F. Levels for IL-1β and IL-6 did not produce significant or consistent results (data not shown).

Strain characterization and genome sequence

The product *E. faecalis* SY is a combination of 10 subcultures of *E. faecalis* strains, that are all highly similar (sometimes denoted S01 to S10). They were originally isolated from the stool of a healthy individual. Two of these have such a high resemblance to starter culture strains of *E. faecalis*, that it is assumed the individual had been colonised with a diary strain; the eight other subcultures all resemble each other and are only slightly different from the other two. The strains are all free of plasmids.

Two genome sequences have been obtained, representative of the clones that form the minority (*Domann* et al., 2007, *Fritzenwanker* et al., 2013), and a third sequencing project is underway that covers two other clones, including one of the majority type. Domann and colleagues presented their genome

sequence in 702 contigs, but the sequence has not been submitted to public databases. In their publication they compared the strain to clinical isolate *E. faecalis* V583 (a well-researched VanA-resistant blood isolate), whose genome was used as a template for assembly (*Domann* et al., 2007). Notably, two gaps were identified representing sequences present in V583 but absent in the *E. faecalis* SY genome. Since these two regions had an aberrant GC content, they were interpreted as genome islands obtained by V583 by horizontal DNA uptake. A number of virulence genes are found in these regions in V583 that are absent in *E. faecalis* SY, such as cytolysin (a toxin that disrupts membranes of other bacteria, erythrocytes and other eukaryotic cells), gelatinase (a secreted bacterial metallo-proteinase that catabolises gelatine, collagen, fibrinogen, casein and insulin, helping the bacteria to spread in the host), hyaluronidase (an enzyme allowing the use of hyaluronic acid as a carbon source), and enterococcal surface protein (assisting in adherence). Since the sequence of *E. faecalis* SY was not fully covered, absence of cytolysin and gelatinase was confirmed by PCR analysis (*Domann* et al., 2007). The *E. faecalis* SY genome does, however, contain the genes coding for aggregation substance (AS, coded by *agg*) and collagen adhesion factor (*ace*), both involved in binding to abiotic surfaces and to eukaryotic cells, and associated with virulence in pathogenic *E. faecalis* strains. These and other genes would support the ability to colonise the intestinal epithelium. Although it was described in the publication that the bacteria were capsulated (which had been postulated for some strains of *E. faecalis* SY [*Kropec* et al., 2005]), the genes responsible for capsulation were not found in the obtained genome sequence. Furthermore, it was determined that the sequenced strain was unable to form biofilms *in vitro* (*Domann* et al., 2007).

In 2013, the complete genome sequence of *E. faecalis* DSM 16431 was released, and although it was not mentioned in the genome announcement, this represents the same strain that Domann and colleagues had sequenced (*Fritzenwanker* et al., 2013). The complete circular genome (GenBank accession nr. HF558530.1) measures 2,810,675 bp, bearing 2733 recognized protein-coding sequences, 4 rRNA loci and 63 tRNAs. The sequence confirmed absence of the *vanB* operon and the pathogenicity island previously noticed by Domann and co-workers. Absence of capsule genes was also confirmed, as well as absence of gene *cas* (an essential gene for functional Cas-CRISPR activity). A unique integrated bacteriophage sequence of 45 kb was recognized that is absent in reference genome V583 (*Fritzenwanker* et al., 2013). *In silico* MLST confirmed the sequence type (ST) of the sequenced strain as ST248. Recently, the draft genome sequences of two other subcultures of *E. faecalis* SY were obtained; DSM16434 resembles DSM16431, while the DSM16430 represents one of the eight other subcultures that are all highly similar to each other. The notable difference between DSM16430 compared to the other two is that it lacks the gene for aggregation substance, and that the prophage is absent (*Fritzenwanker* et al., 2016).

Safety and colonisation aspects of *Enterococcus faecalis* SY

An *in vitro* opsonophagocytic test (the capacity of PMNs to take up and kill bacteria in presence of serum) was used to compare the serum resistance of *E. faecalis* SY bacteria with other *Enterococcus* strains, as a measure for safety of the product. This demonstrated that

E. faecalis SY is serum sensitive (*Kropec* et al., 2005).

Since virulent *E. faecalis* strains are frequently causing urinary tract infections (UTIs), in particular in nosocomial settings related to catheter use, the ability of various strains, including *E. faecalis* SY, to multiply in urine was assessed (*Vebø* et al., 2010). Division time did not vary much between *E. faecalis* SY and pathogenic strains, though the latter reached higher cell densities in urine ($2x10^8$ compared to $1.2x10^8$ CFU/ml for *E. faecalis* SY). Transcriptional analysis of bacteria exposed to urine for 30 minutes was performed by microarray analysis (the array did not contain *E. faecalis* SY-specific genes). The results indicated the bacteria had switched to alternative carbohydrate metabolism since glucose levels in urine are low, as well as other adaptations. In most of the described observations, *E. faecalis* SY behaved similar to the pathogenic strains MMH594 and OG1RF. This study was not conducted to specifically compare probiotic *E. faecalis* with virulent strains, but one observation was noticeable according to the authors: gene EF3314 was only upregulated in *E. faecalis* SY and not in the other two strains as a result of urine exposure. They described the product of EF3314 as a potential substrate for sortase A (coded by *srtA*, EF3056, also upregulated), which is important for adherence to abiotic surfaces and biofilm formation, and is considered a virulence gene for *E. faecalis* (*Vebø* et al., 2010). Comparative genome hybridization (CGH) was also performed to compare the three strains. As expected, variation was observed in mobile elements mainly. Of interest is also the observation that serotype 2 capsular polysaccharide genes (*cps*) were absent in the *E. faecalis* SY strain they used, which may explain why these bacteria are sensitive to phagocyte killing. From this observation it is likely they worked with S01. The MLST sequence type of *E. faecalis* SY was reported to be CC25, ST248, in accordance to the sequence type that Fritzenwanker and colleagues had obtained.

Hoffmann and colleagues investigated the ability of *E. faecalis* to cause colitis, using a knock-out mouse deficient of IL-10, which renders the animals susceptible to chronic intestinal inflammation in response to certain *E. faecalis* strains (*Hoffmann* et al., 2011). In a healthy host, the inflammatory response to bacteria present in the gut is tightly regulated so that non-pathogens are safely ignored (though monitored), but when this regulation is faulty, chronic inflammation is the unwanted outcome. A mouse model in which the 'safety valve' IL-10 is knocked out is used as a model to elucidate the causes of Crohn's disease (CD) and inflammatory bowel disease (IBD) in humans. When *E. faecalis* SY was compared *in vitro* to the colitogenic control strain OG1RF, both induced IL-6 and IFN-γ production in a non-differentiated murine cell line (*Hoffmann* et al., 2011). These findings are in line with those previously reported by *Rosenkranz* and *Grundmann* (1994). Both strains triggered pro-inflammatory responses via the pattern recognition receptor TLR-2 (*Hoffmann* et al., 2011). The probiotic bacteria also resulted in pro-inflammatory epithelial cell activation *in vivo*, but only when tested in germfree IL-10-deficient mice; wild-type animals did not develop disease in response to *E. faecalis* SY or the colitogenic strain (*Hoffmann* et al., 2011). It should be pointed out, that *E. faecalis* probiotics can reduce colitis symptoms in IL-10-deficient mice suffering from colitis when they are not raised germ-free. The authors admit that missing IL-10 is an immense immunological deficiency,

and that their mouse model is rather simplistic compared to human IBD.

Using a number of *in vitro* tests, Christoffersen and colleagues compared *E. faecalis* SY with four other *E. faecalis* strains: pathogenic V583 (the well-researched VanA-resistant clinical isolate), two commensal faecal isolates from babies (strains 62 and 158B) and a cheese strain called LMGT3208 (*Christoffersen* et al., 2012). After 30 min exposure to human gastric juice of pH 1.5, viability of all strains was severely impaired, and even when the pH was artificially raised to 5, exposure to the digestive enzymes present in gastric juice damaged all 5 strains. However, *E. faecalis* SY was least capable to tolerate acid exposure, as it grew poorly in BHI of pH 4.5 compared to the other strains. Exposure to human duodenal juice for 30 minutes also affected viability, but in this test *E. faecalis* SY survived better (91% survival) than the other four strains. Adherence to Caco-2 cells or mucin was significantly weaker for *E. faecalis* SY than for the others. Binding to glycosaminoglycans (host cell receptors) or inducing dendritic cell (DC) maturation was similar for all strains (*Christoffersen* et al., 2012). These results would suggest that *E. faecalis* SY is not well equipped to survive in the human stomach.

The capability to survive passage through the gastrointestinal tract was further assessed using two *in vitro* models to resemble passage through an empty stomach as well as a full stomach (*Wassenaar* et al., submitted for publication). Again, *E. faecalis* SY was rapidly inactivated by gastric pH, though sufficient bacteria survived to produce stable numbers in conditions subsequently resembling the small intestine. Nevertheless, a single dose did not result in intestinal colonisation, when tested with a single volunteer: live *E. faecalis* SY were only detected in stool for a few days following a single dose (*Wassenaar* et al., submitted for publication). This could be demonstrated with the use of strain-specific primers that had been designed by use of the genome sequence.

In view of the functionality of *E. faecalis* SY to treat recurrent upper respiratory tract infections and sinusitis, it was tested if the bacteria were able to colonise the throat instead. Two volunteers gurgled with a single dose of the product for 30 seconds, after which the product was swallowed. Immediately thereafter, live bacteria could be detected from swabs of the mouth and throat of both individuals, but not any more two hours later, or at any other time point tested up to 24 hrs. (C. Beimfort and K. Zimmermann, unpublished observations). It has not yet been established if prolonged exposure with multiple doses results in intestinal colonisation, or in elevated saliva IgA levels in humans.

ESCHERICHIA COLI PREPARATIONS

Animal studies with *E. coli* SY

The 1983 publication already cited for mouse experiments with *E. faecalis* SY also described how 4 weeks of oral administration of *E. coli* SY provided a similar level of protection against intraperitoneal challenge with *H. influenzae* (*Rusch* et al., 1983). Similar results were published a year later, when it was described that the LD_{50} increased from an intraperitoneal dose requiring a $10^{-6.0}$ dilution of challenge *H. influenzae* (no probioticum) to a dilution of $10^{-3.8}$ when the challenge followed 3 weeks of *E. coli* administration (*Rusch* et al., 1984).

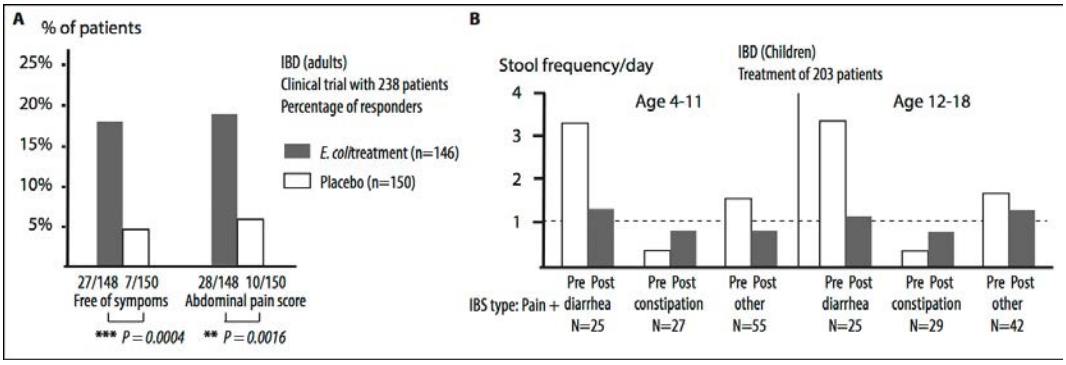

Figure 6: Treatment of IBD with *E. coli* SY.
Panel A shows the key outcome of a clinical trial involving 238 adult IBD patients. (After *Enck* et al., 2009). Panel B shows improved stool frequency in two age groups of paediatric IBD patients. (Modified after *Martens* et al., 2010).

As with *E. faecalis*, the mode of action of probiotic *E. coli* is most likely via immune-modulation. A key publication in this respect is from 2008, which describes the function of mast cells as the policemen of the immune system (*Magerl* et al., 2008). When these cells encounter pathogenic bacteria, they release pre-synthesized substances to recruit lymphocytes and dendritic cells, whereby they respond differently to non-pathogenic bacteria. In response to the latter, mast cells produce IL-15, which tunes down the recruitment of immune cells, together with a collection of other genes that are up- or down regulated during the process. An *in vitro* model using murine mast cells was applied to investigate how these cells respond to *E. coli* SY (*Magerl* et al., 2008). When pre-incubated with the bacteria, the mast cells were no longer triggered by a calcium ionosphere or an IgG/allergen combination. The observed inhibition of degranulation was concentration-dependent in a binary manner, with little effect observed below 15,000 bacteria per mast cell, but no further increase at higher inoculates. Sterile culture supernatant or paraformaldehyde-killed bacteria did not inhibit degranulation. When mice were injected intraperitoneally with a suspension of the probiotic bacteria, mast cells harvested a day later from the peritoneum were again less responsive to the tested triggers. By varying the time between *i.p.* dosage and mast cell harvest, it was established that the effect faded over the course of a few days (*Magerl* et al., 2008). As a side note, it should be mentioned that the mice did not suffer from the bacterial load and survived the treatment till the end of the experiments.

Clinical trials with *E. coli* SY

A randomized double-blind clinical trial was published in which *E. coli* SY was tested for treatment of IBD, involving 238 patients. Treatment lasted for 8 weeks, The key finding was that there were significantly more responders that became free of symptoms (27 out of 148) than in the placebo group (7/150) (*Enck* et al., 2009). The percentages for patients with lower abdominal pain scores were also significantly different. The results are summarized in Figure 6, Panel A. The trial was included in a systematic review that was performed to assess probiotic therapies in adult IBS patients, which covered 37 publications in total (*Hungin* et al., 2013). The majority of

the compared studies focused on IBS with diarrhoeal symptoms. The results obtained with *E. coli* SY were cited in 5 of 16 statements by Hungin and colleagues: nr. 1, [the product] help(s) relieve overall symptom burden; nr. 4, helps reduce abdominal pain; nr. 5, helps reduce bloating/distension; nr. 8, helps improve frequency and/or consistency of bowel movements; and nr. 12, improvement of symptoms leads to improvement in some aspects of health-related quality of life (*Hungin* et al., 2013).

Treatment with *E. coli* SY was also tested in 203 paediatric IBD patients (age range 4-11 and 12-18 years), although this was not a blinded or placebo-controlled study (*Martens* et al., 2010). The children were treated for 43 days on average and this treatment was well tolerated. The stool frequency improved, both for IBD in combination with diarrhoea as for constipated patients (Figure 6B). Pain also decreased significantly in both age groups that were analysed.

Mechanistic explanations

Early work by Jansen and co-workers had shown how intake of *E. coli* SY changed levels of immunoglobulins (*Jansen* et al., 1998). Ten healthy volunteers donated two serum samples (3 weeks apart) prior to a 2-weeks daily intake of the product, after which another serum sample was taken, with a follow-up 4 weeks later. Faecal samples were collected on a weekly basis during the complete investigation. Serum IgG, IgM and IgA levels were tested for binding capacity to *E. coli* SY, whereby the individual's levels prior to intake of the product served as an internal control. This work demonstrated an increase in IgG serum levels only, for all 10 individuals, that lasted throughout the follow-up period (*Jansen* et al., 1998).

That *E. coli* induces production of epithelial β-defensins in the human host provides a mechanistic explanation of beneficial effects of *E. coli* SY. After 23 healthy volunteers had taken the product for 3 weeks, their stool samples contained elevated levels of human β-defensin-2 (hBD-2), as determined by ELISA, in contrast to 5 volunteers taking placebo (*Möndel* et al., 2009).

The ability to inhibit the growth of pathogenic species, as in the originally proposed mechanism of probiotic *E. coli* by Alfred Nissle, was investigated by comparing *E. coli* Nissle 1917 and a number of commensal isolates for their ability to inhibit growth of Shiga-toxin producing *E. coli* (STEC) (*Reissbrodt* et al., 2009). In comparison to *E. coli* Nissle and a serendipitously found commensal strain, which both reduced Shiga toxin levels with over 90% during co-cultivation, *E. coli* SY could only reduce these levels with 10%, whereas other strains and *E. faecalis* SY bacteria had no effect at all (*Reissbrodt* et al., 2009).

Novel insights on the mechanism of probiotic action were obtained with the discovery of Microcin S (*Zschüttig* et al., 2012). This novel type of bacteriocin is only produced by *E. coli* SY genotype G3/10, transcribed from an operon of 4 genes present on the megaplasmid of this isolate. *In vitro* activity of the microsin against an enteropathogenic *E. coli* strain (EPEC) was demonstrated, and the authors speculated on the use of this novel bacteriocin as a potential antitumor agent (*Zschüttig* et al., 2012). According to the scheme of Cotter and colleagues, Microcin S belongs to class IId (unmodified anacyclamides) (*Cotter* et al., 2013).

Zschüttig and colleagues speculated on the use of this novel bacteriocin as a potential antitumor agent. The idea was

further investigated in a recent publication (*Kocijancic* et al., 2016). In mouse experiments it was demonstrated that the Lux-marked G1/2 strain of *E. coli* SY specifically target tumours after intravenous administration. Whereas bacteria rapidly reached liver and spleen, from which they were cleared, tumours were colonised more slowly but persistently (*Kocijancic* et al., 2016). This line of work could open future perspectives of the product as a delivery vehicle for anti-tumour therapeutics.

Strain characterization and genome sequences of *E. coli* SY

The 10 subcultures that make up *E. coli* SY can be divided into 4 or 6 genotypes, depending on the sensitivity of the typing method applied. These are called G1/2 (20% in the final product), G3/10 (20%), G4/9 (20%), G6,7 (20%), G5 (10%), and G8 (10%). The strains G1/2, G6/7 and G8 contain the same two plasmids and share a number of other characteristics (see below), so that they are sometimes regarded as representatives of one and the same genotype. All originate from the stool of a healthy volunteer.

The first genetic characterisation was performed by microarray analysis, using a microarray based on 24 *E. coli* and 8 *Shigella* genome sequences that were available at that time (*Willenbrock* et al., 2007). The analysis was performed with G1/2, G3/10, G4/9 and G5 (G6/7 and G8 were omitted), resulting in the first detailed insights about the genetic make-up of these types. The number of predicted genes varied from 3568 (G4/9) to 3978 (G1/2), and the four genotypes shared marginally more genes with commensal strain *E. coli* K-12 MG 1655 than with enteropathogenic strain EDL933. A hierarchical cluster analysis of the hybridization signals revealed that the genomes of the *E. coli* SY components more closely resembled K12 than EDL933 or other pathogenic strains. A core genome of 3083 genes was identified that all four genotypes shared (*Willenbrock* et al., 2007). The authors further reported presence of a haemolysin operon (*hlyABCD*) in genotype G1/2, in accordance to the weakly haemolytic phenotype of this isolate. Genotypes G6/7 and G8 also contain this operon.

The genome sequences of the 6 genotypes were released in 2015, accompanied by a genome announcement publication (*Zschüttig* et al., 2015). The chromosome of G3/10 was assembled to completion, while the other genotypes were published as multiple contigs. The plasmids of the strains, varying in number from 1 (G4/9) to 6 (G3/10) were also sequenced.

Safety and colonisation aspects of *E. coli* SY

The haemolysin gene was not the only virulence gene detected in these strains; other genes that are typically associated with pathogenic *E. coli* were found present as well. These were described and discussed in a publication that concentrated on the safety of the product (*Wassenaar* et al., 2015). It was concluded that presence of individual virulence genes cannot be taken as evidence for pathogenicity per se. Combined with the ten-years long collation of all side effects collected from commercial use, which was a surprisingly short list, it was concluded that the product was safe for human use (*Wassenaar* et al., 2015). This conclusion was further substantiated in a discussion paper where evidence from other *E. coli* strains was also included, to support the view that presence of virulence genes is not always a sign of a pathogenic lifestyle: the genomic background determines to a large extent the phenotype of a strain of *E. coli*

(*Wassenaar* and *Gunzer*, 2015).

The genome sequences were used to develop strain-specific probes, which enabled the specific detection of *E. coli* SY components. These were used to determine the colonisation potential of the product in humans after a single dose. (*Wassenaar* et al., 2014). It turned out that the strains colonised well: all volunteers were colonised by *E. coli* SY for at least 12 weeks and for two individuals the product could be detected for 27 weeks. This was demonstrated by colony-lift hybridization from stool cultures. During the first week following the single dose intake, the detected genotypes fluctuated, but after a few weeks all but one of the genotypes had disappeared. In all volunteers only genotype G1/2, which could not be distinguished from G6/7 and G8, survived (*Wassenaar* et al., 2014). Not all volunteers took the same dose, and by comparison of the highest and lowest dose, which were a factor of 10 apart, it was deduced that the persistence of these three genotypes (which together comprise 50% of the product) was not the result of a numerical advantage in the dose.

By use of dynamic *in vitro* models it was established that *E. coli* SY were decimated during gastric passage, but enough bacteria survived to multiply as soon as conditions mimicking the jejunum were applied (*Wassenaar* et al., submitted for publication). It has not yet been established if dominant colonisation of the genotype G1/2 would also result if this strain was administered by itself, or whether an interplay with the other genotypes creates a local niche, which subsequently enhances growth of G1/2.

BACTERIAL LYSATE PSY

Animal studies with the bacterial Lysate PSY

The effectivity of Lysate PSY to alleviate immunological overreaction was first modelled for food allergy by use of a rat model (*Ahrens* et al., 2011). Rats were sensitized with intraperitoneal ovalbumin (OVA) to trigger an allergic reaction, after which the animals were orally challenged with OVA, and humoral antibody levels were determined. One group of animals received Lysate PSY prior to and during sensitization, while the control group did not receive the lysate. Sensitization resulted in a 75% increase of total IgE levels in both groups, but while OVA-specific IgE was absent in non-sensitized animals, these levels were much lower in the treated animals than in the control group; similar results were recorded for IgG (*Ahrens* et al., 2011). Mononuclear cells (MNCs) in the spleen were isolated post-mortem and these were stimulated with OVA *in vitro*. The cells from sensitized animals responded stronger than cells from non-sensitized animals, but not when the animals had been treated with the bacterial lysate. When the cytokines produced by spleen MNCs were compared, the cells isolated from the treated rats produced significantly more IL-10 than the cells isolated from control animals. An even stronger difference was seen with MNCs isolated from the mesenteric lymph nodes. All these results suggested that the treatment with Lysate PSY had dampened the immune response to the ovalbumin allergen ovalbumin (*Ahrens* et al., 2011).

Clinical studies with Lysate PSY

A clinical trial that was performed with Lysate PSY in 1988-1989 was originally published in German (*Panijel* and *Burkhard*, 1993) and later re-analysed

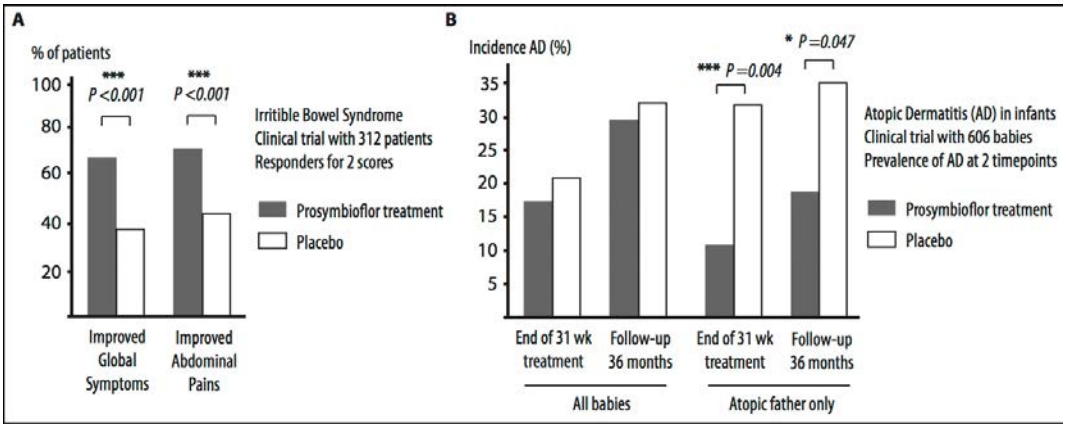

Figure 7: Results from two clinical trials with bacterial Lysate PSY.
Panel A shows the percentage of responders in a clinical trial involving 312 adult IBS patients who were treated for 8 weeks, based on two scores. (Modified after *Enck* et al., 2008). Panel B shows a trial with 606 babies with atopic dermatitis (AD) as the measured outcome, determined after 31 weeks of treatment and at a follow-up time-point of 36 months. There was a significant difference only for a subgroup of patients whose father had a history of atopic disease. (After *Lau* et al., 2012).

and presented in English (*Enck* et al.,2008). The reason for the re-analysis was that when the trial was originally described, neither the Rome criteria for the diagnosis of IBS, nor the FDA and EMA guidelines for analysis of clinical trials had been available, which were subsequently applied. The double-blinded trial involved 312 adult IBS patients who suffered from abdominal pain. Of these, 297 were randomly divided into a placebo and treatment group. Treatment lasted for 8 weeks. During the trial 18 patients dropped out, 5 (3 in the treatment and 2 in the placebo group) because of adverse effects. The outcome of treatment was recorded using a global symptom score (GGS) and an abdominal pain score. Based on the GGS, 102 of the 149 patients (68.5%) receiving Lysate PSY were considered as drug responders, meaning the symptom score had significantly decreased. This was a highly significant difference compared to the 37.8% responders receiving placebo, visualized in Figure 7, Panel A. A similar positive result was obtained for abdominal pain scores, with 72.5% responders in the treatment and only 44.6% in the placebo group. The authors concluded that although there is no mechanistic explanation how bacterial lysates can relieve symptoms in IBS patients, such products may have lower adverse events and a higher acceptance in patients compared to classical probiotic products based on live bacteria (*Enck* et al., 2008).

A meta-analysis published a year later included the results of the Enck study, though it cited a meeting abstract as the source, because the complete publication had not yet been available when the meta-analysis was performed (*McFarland* and *Dublin*, 2008). In the resulting forest plot, the Enck study showed a favourable effect with an relative risk of 0.51 (95% confidence interval 0.39-0.66) of relative risk for IBS symptoms after probiotic treatment compared to placebo. Note that in this way of expressing the data, an relative risk <1 is indicative of a therapeutic effect. With this score, Lysate PSY was amongst the better-

producing microbiologics, while the findings had a small 95% confidence interval.

Another clinical trial was directed towards prevention of atopic dermatitis in children (*Lau* et al., 2012). The double-blind, randomized placebo-controlled trial involved infants with one or two atopic parents as well as children without such a genetic history. A total of 606 babies were enrolled, half of who received Lysate PSY from week 5 till 7 months of age. The children were followed until they reached the age of 3 (*Lau* et al., 2012). The number of children not completing the treatment, as well as number and severity of adverse effects, were not significantly different between treatment and control group. Disappointingly, when the complete study group was analysed, treatment and control group apparently were apart, but they did not significantly differ in frequency of atopic dermatitis (Figure 7B). This was also true for the three subgroups of children who had two atopic parents, children with parents who were both not atopic, and children with an atopic mother (*Lau* et al., 2012). However, the prevalence of atopic dermatitis was significantly lower ($p<0.004$) in the treatment group compared with controls for children whose father but not the mother was atopic. This difference was obvious at the end of the intervention phase (week 31) but tailed off to a borderline significance ($p<0.047$) at 36 months. The authors stated they were aware that subgroup analysis in a clinical trial must be interpreted with caution. One should not analyse towards a desired effect, but in this case it was known that prevalence of atopic dermatitis is in part related to the atopic state of the parent(s). Although the activity of LPS in the gut can serve as a mechanistic explanation, the difference in protection for atopic fathers versus no protection for atopic mothers cannot be explained. Before speculating on underlying mechanisms at play here, more data would be needed; not only on the mechanism of action of Lysate PSY but also a confirmation of the trial findings with an independent study.

A year later, the topic was revisited by a new analysis of the same infants cohort. During the trial, stool samples of the children had been collected at week 5 (when the Lysate PSY intervention started), at week 13 and at week 31, when intake of the lysate was terminated. The microbiome of these stool samples were investigated by culture-dependent methods and by DNA-dependent methods. (*Penders* et al., 2013). The results provided some valuable insights in the composition of the microbiota of an infant. First of all, whether the babies had received Lysate PSY or placebo made no difference, which was to be expected, since the lysate does not contain viable bacteria. This observation rules out the possibility that any clinical effect of Lysate PSY is the indirect result in shifts of intestinal microbiota (in theory, the microbiota could shift as a result of the bacterial lysate, or by induced local immune-modulation, but neither seemed to be the case). However, as had been observed before, birth mode (vaginal versus caesarean birth) had a profound effect on microbiota composition (*Penders* et al., 2013). Surprisingly, another independent and strong influence was found for birth order: with increasing number of older siblings, the number of *Lactobacillus* and *Bacteroides* species increased, while frequency of Clostridiales decreased (*Penders* et al., 2013). Especially this later finding is relevant, as the risk of developing atopic dermatosis increases with *Clostridium* colonisation, and this was found in the cohort under study as well, in particular for members of *Clos-*

tridium cluster I. No effect on composition of the intestinal composition was reported for day care attendance or maternal and paternal atopy.

This is not to say that early administration of Lysate PSY can prevent other atopic symptoms. The same study groups were analysed at age 6-11 years for prevalence of asthma or rhinoconjunctivitis, for which no significant difference between treatment and placebo group could be established (*Rossberg* and colleagues, unpublished data).

The economic burden of atopic dermatitis and the cost-effectiveness of possible prevention by Lysate PSY were calculated by *Kiencke* et al. (2013). It was calculated that preventive treatment with Lysate PSY was cost effective at 3 years and at 6 years for children with one atopic parent ('single heredity for atopy'). The effectiveness of costs would probably be even higher for children with an atopic father.

Product characterization and quality control of Lysate PSY

Lysate PSY is produced by separate culture of the bacterial components *E. faecalis* SY and *E. coli* SY to approximately $3x10^7$ CFU/ml, after which the bacterial cultures are cooled to 6˚C. The cultures are then combined and sterilized at 121˚C for 20 minutes under constant pressure. The result is a clear solution, which is bottled in a sterile environment.

Concerns about variable content and lack of quality control of a bacterial lysate were minimised by a study by Klein and co-workers, who used a proteomics approach to determine the quality of Lysate PSY (*Klein* et al., 2013). By analysis of the total protein content, the complexity of the components present in a bacterial lysate can be assessed qualitatively as well as quantitatively. The authors used a high-throughput, high-resolution technique in which capillary electrophoresis was coupled to mass spectrometry (CE/MS). The output is presented in the form of three-dimensional contour plots. These capture the migration time of electrophoresis, the mass (on a logarithmic scale) of detected peptides in kDa and the signal intensity as a quantitative measure (*Klein* et al., 2013). The results showed that various lots of Lysate PSY were of relatively constant qualitative constitution, although the data would have been more convincing if a deliberate aberrant sample (e.g. the separate lysates of *E. faecalis* and *E. coli*) were also included for comparison. The major differences reported between lots were quantitative, as some individual peak heights varied between batches (*Klein* et al., 2013). It must be noted, that peptides were also detected in sterile culture media, though there were 3 times more different peptides found in the bacterial lysate. The major peptides were recovered and sequenced, which identified that 517 peaks were derived form *E. faecalis* proteins and 406 were from *E. coli* (*Klein* et al., 2013). Interestingly, whereas the majority of peptides from *E. coli* proteins originated from cell membrane and fimbriae, those of *E. faecalis* were mostly cytosolic.

CONCLUSIONS

The data presented in this paper allow the conclusion that symbiotic bacteria offer a great therapeutic potential. Furthermore the results of multidisciplinary investigations around bacterial products based upon Symbio *E. fae-*

calis SY and *E. coli* SY open possibilities to hitherto unexpected medical targets. This will require many more research efforts. There is no doubt, however, that the use of microbiologic or probiotic drugs containing live *Enterococcus faecalis* or live *Escherichia coli* or a bacterial lysate thereof is safe. In numerous experimental *in vitro* and animal studies, as well as by human clinical studies, efficacy of such microbiologics or probiotics was established. The mode of action is apparent in modulation of many physiologic parameters and most prominently in modulation of immune activities.

IDENTIFICATION OF BACTERIAL PRODUCTS DESCRIBED IN THIS PUBLICATION

The bacterial preparations described in this paper are produced by SymbioPharm GmbH, Herborn, Germany. We use the following abbreviations for the products of SymbioPharm: *E. faecalis* SY describes Symbioflor 1 containing live *Enterococcus faecalis (DSM 16440)*, *E. coli* SY is used for Symbioflor 2 containing live *Escherichia coli (DSM 17252)*, and Lysate PSY describes Pro-Symbioflor, a bacterial lysate manufactured from *Enterococcus faecalis (DSM 16440)* and *Escherichia coli (DSM 17252)*.

LITERATURE

Ahrens, B., Quarcoo, D., Buhner, S., Matricardi, P.M., and Hamelmann, E.: Oral administration of bacterial lysates attenuates experimental food allergy. Int. Arch. Allergy Immunol. 156, 196-204 (2011).

Christoffersen, T.E., Jensen, H., Kleiveland, C.R., Dørum, G., Jacobsen, M., and Lea, T.: In vitro comparison of commensal, probiotic and pathogenic strains of Enterococcus faecalis. Br. J. Nutr. 108, 2043-2053 (2012).

Cotter, P.D., Ross, R.P., and Hill, C.: Bacteriocins - a viable alternative to antibiotics? Nat. Rev. Microbiol. 11, 95-105 (2013).

Domann, E., Hain, T., Ghai, R., Billion, A., Kuenne, C., Zimmermann, K., and Chakraborty, T.: Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic Enterococcus faecalis strain Symbioflor 1. Int. J. Med. Microbiol. 297, 533-539 (2007).

Enck, P., Zimmermann, K., Menke, G., Müller-Lissner, S., Martens, U., and Klosterhalfen, S.: A mixture of Escherichia coli (DSM 17252) and Enterococcus faecalis (DSM 16440) for treatment of the irritable bowel syndrome--a randomized controlled trial with primary care physicians. Neurogastroenterol. Motil. 20, 1103-1109 (2008).

Enck, P., Zimmermann, K., Menke, G., and Klosterhalfen, S.: Randomized controlled treatment trial of irritable bowel syndrome with a probiotic *E.-coli* preparation (DSM17252) compared to placebo. Z. Gastroenterol. 47, 209-214 (2009). Erratum in Z. Gastroenterol. 52, 64 (2014).

Fritzenwanker, M., Kuenne, C., Billion, A., Hain, T., Zimmermann, K., Goesmann, A., Chakraborty, T., and Domann, E.: Complete Genome Sequence of the Probiotic Enterococcus faecalis Symbioflor 1 Clone DSM 16431. Genome Announc. 1, e00165-12 (2013).

Fritzenwanker, M., Chakraborty, T., Hain, T., Zimmermann, K., and Domann, E.: Draft Genome Sequence of the Probiotic Enterococcus faecalis Symbioflor 1 Clones DSM 16430 and 16434. Genome Announc. 4, e01061-16 (2016).

Habermann, W., Zimmermann, K., Skarabis, H., Kunze, R., and Rusch, V.: Einfluß eines

bakteriellen Immunstimulans (humane Enterococcus faecalis-Bakterien) auf die Rezidivhäufigkeit bei Patienten mit chronischer Bronchitis. Arzneimittelforschung 51, 931-937 (2001).

Habermann, W., Zimmermann, K., Skarabis, H., Kunze, R., and Rusch, V.: Verminderung der Rezidivhäufigkeit bei Patienten mit chronisch rezidivierender hypertrophischer Sinusitis unter Behandlung mit einem bakteriellen Immunstimulans (Enterococcus faecalis-Bakterien of humaner Herkunft). Arzneimittelforschung 52, 622-627 (2002).

Hoffmann, M., Messlik, A., Kim, S.C., Sartor, R.B., and Haller, D.: Impact of a probiotic Enterococcus faecalis in a gnotobiotic mouse model of experimental colitis. Mol. Nutr. Food Res. 55, 1-11 (2011).

Hungin, A.P., Mulligan, C., Pot, B., Whorwell, P., Agréus, L., Fracasso, P., Lionis, C., Mendive, J., Philippart de Foy, J.M., Rubin, G., Winchester, C., de Wit, N.; European Society for Primary Care Gastroenterology: Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice -- an evidence-based international guide. Aliment. Pharmacol. Ther. 38, 864-886 (2013).

Hyde, R.M., Chan, G., and Rusch, V.: Oral vaccination enhances phagocytic functions. Microecol. Ther. 16, 297-301 (1986).

Jansen, G., Deddens, B., Wilkinson, M., and van der Waaij, D.: Significant decrease of titres of circulating IgG after oral intake of a preparation of Enterococcus faecalis in a group of ten healthy volunteers. Infection 21, 193-194 (1993).

Jansen, G.J., Wildeboer-Veloo, A.C., van der Waaij, D., and Degener, J.E.: Escherichia coli as a probiotic? Infection 26, 232-233 (1998).

Kalinski, S.: Steigerung der körpereigenen Abwehr bei chronisch rezidivierender Tonsillitis. Fortschr. Med. 104, 843-846 (1986).

Kalinski, S.: Immunestimulation bei Infektionen der oberen Luftwege und im HNO-Bereich. Klinische Prüfung eines Streptokokken-Präparates. Therapiewoche 37, 3371-3378 (1987).

Kiencke, P., Viehmann, K., and Rychlik, R.: Cost-effectiveness analysis, prevention of atopic dermatitis by oral application of bacterial lysate in newborns/small children. Eur. J. Health Econ. 14, 995-1002 (2013).

Kitz, R., Martens, U., Zieseniß, E., Enck, P., and Rose, M.A.: Probiotic E. faecalis – adjuvant therapy in children with recurrent rhinosinusitis. Centr. Eur. J. Med. 7, 362-365 (2012).

Klein, G., Schanstra, J.P., Hoffmann, J., Mischak, H., Siwy, J., and Zimmermann, K.: Proteomics as a Quality Control Tool of Pharmaceutical Probiotic Bacterial Lysate Products. PLoS One 8, e66682 (2013).

Kocijancic, D., Felgner, S., Frahm, M., Komoll, R.M., Iljazovic, A., Pawar, V., Rohde, M., Heise, U., Zimmermann, K., Gunzer, F., Hammer, J., Crull, K., Leschner, S., and Weiss, S.: Therapy of solid tumors using probiotic Symbioflor-2: restraints and potential. Oncotarget. 7, 22605-22622 (2016).

Kropec, A., Hufnagel, M., Zimmermann, K., and Huebner, J.: In vitro assessment of the host response against Enterococcus faecalis used in probiotic preparations. Infection 33, 377-379 (2005).

Lau, S., Gerhold, K., Zimmermann, K., Ockeloen, C.W., Rossberg, S., Wagner, P., Sulser, C., Bunikowski, R., Witt, I., Wauer, J., Beschorner, J., Menke, G., Hamelmann, E., and Wahn, U.: Oral application of bacterial lysate in infancy decreases the risk of atopic dermatitis in children with 1 atopic parent in a randomized, placebo-controlled trial. J. Allergy Clin. Immunol. 129, 1040-1047 (2012).

Magerl, M., Lammel, V., Siebenhaar, F., Zuberbier, T., Metz, M., and Maurer, M.: Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells. Exp. Dermatol. 17, 427-435 (2008).

Martens, U., Enck, P., and Zieseniss, E.: Probiotic treatment of irritable bowel syndrome in children. Ger. Med. Sci. 8, Doc07 (2010).

McFarland, L.V. and Dublin, S.: Meta-analysis

of probiotics for the treatment of irritable bowel syndrome. World J. Gastroenterol. 14, 2650-2661 (2008).

Möndel, M., Schroeder, B.O., Zimmermann, K., Huber, H., Nuding, S., Beisner, J., Fellermann, K., Stange, E.F., and Wehkamp, J.: Probiotic E. coli treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol. 2, 166-172 (2009).

Nissle, A.: Über die Grundlagen einer neuen ursächlichen Bekämpfung der pathologischen Darmflora. Dtsch. Med. Wochenschr. 42, 1181-1184 (1916).

Ottendorfer, D., Zimmermann, K., Taborski, B., Thoma, W., Thoma, H., and Schade, I.: Immunologische Ex-vivo Untersuchungen zum Wirkmechanismus von humanen Enterococcus faecalis-Bakterien (Symbioflor 1®) an Miniaturschweinen. Forsch. Komplementmed. 2, 302-309 (1995).

Panijel, M. and Burkhard, I: ProSymbioflor zur Behandlung des irritablen Kolons. Jatros Naturheilkunde 2, 1-4 (1993).

Penders, J., Gerhold, K., Stobberingh, E.E., Thijs, C., Zimmermann, K., Lau, S., and Hamelmann, E.: Establishment of the intestinal microbiota and its role for atopic dermatitis in early childhood. J. Allergy Clin. Immunol. 132, 601-607 (2013).

Reissbrodt, R., Hammes, W.P., dal Bello, F., Prager, R., Fruth, A., Hantke, K., Rakin, A., Starcic-Erjavec, M., and Williams, P.H.: Inhibition of growth of Shiga toxin-producing Escherichia coli by nonpathogenic Escherichia coli. FEMS Microbiol. Lett. 290, 62-69 (2009).

Rosenkranz, W. and Grundmann, E.: Immunmodulatorische Wirkung lebender nichtpathogener Enterococcus faecalis Bakterien des Menschen. Arzneimittelforschung 44, 691-695 (1994).

Rusch, V. and Zimmermann, K.: Microbial therapy with Enterococcus faecalis and Escherichia coli: experimental and clinical data. In: Probiotics: prospects of use in opportunistic infections. Old Herborn University Seminar Monograph 8 (Eds,: Fuller, R., Heidt, P.J., Rusch, V., and van der Waaij, D.). Institute for Microbiology and Chemistry, Herborn-Dill, Germany, 158-173 (1995).

Rusch, V., Hyde, R.M., and Luckey, T.D.: Application of S. faecalis and E. coli oral vaccines in humans and animals. Prog. Food Nutr. Sci. 7, 39-52 (1983).

Rusch, V., Hyde, R.M., and Luckey, T.D.: Immunostimulation in Tiermodellen mit Hilfe von Präeparaten intestinaler Bakterien. Nahrung 28, 699-709 (1984).

Rusch, V., Kalinski, S., and Schneider, H.: Streptococcus faecalis vaccine - results of double blind placebo studies. Microecol. Ther. 16, 59-74 (1986).

Vebø, H.C., Solheim, M., Snipen, L., Nes, I.F., and Brede, D.A.: Comparative genomic analysis of pathogenic and probiotic *Enterococcus faecalis* isolates, and their transcriptional responses to growth in human urine. PLoS One. 5, e12489 (2010).

Vouloumanou, E.K., Makris, G.C., Karageorgopoulos, D.E., and Falagas, M.E. Probiotics for the prevention of respiratory tract infections: a systematic review. Int. J. Antimicrob. Agents 34, 197.e1-197.e10 (2009).

Wassenaar, T.M. and Gunzerm, F.: The prediction of virulence based on presence of virulence genes in E. coli may not always be accurate. Gut Pathog. 7, 15 (2015).

Wassenaar, T.M., Beimfohr, C., Geske, T., and Zimmermann, K.: Voluntarily exposure to a single, high dose of probiotic Escherichia coli results in prolonged colonisation. Benef. Microbes 5, 367-375 (2014).

Wassenaar, T.M., Zschüttig, A., Beimfohr, C., Geske, T., Auerbach, C., Cook, H., Zimmermann, K., and Gunzer, F.: Virulence genes in a probiotic E. coli product with a recorded long history of safe use. Eur. J. Microbiol. Immunol. (Bp) 5, 81-93 (2015).

Wassenaar, T.M., Marzorati, M., Beimfohr, C., Siegl, A., and Zimmermann, K.: Survival of probiotic E. coli and Ent. faecalis in the human host after oral uptake: results from in vitro and in vivo studies. Submitted for

publication (2016).
Willenbrock, H., Hallin, P.F., Wassenaar, T.M., and Ussery, D.W.: Characterization of probiotic *Escherichia coli* isolates with a novel pan-genome microarray. Genome Biol. 8, R267 (2007).
Zschüttig, A., Zimmermann, K., Blom, J., Goesmann, A., Pöhlmann, C., and Gunzer, F.: Identification and characterization of microcin S, a new antibacterial peptide produced by probiotic Escherichia coli G3/10. PLoS One.7, e33351 (2012).
Zschüttig, A., Auerbach, C., Meltke, S., Eichhorn, C., Brandt, M., Blom, J., Goesmann, A., Jarek, M., Scharfe, M., Zimmermann, K., Wassenaar, T.M., and Gunzer, F.: Complete Sequence of Probiotic Symbioflor 2 Escherichia coli Strain G3/10 and Draft Sequences of Symbioflor 2 E. coli Strains G1/2, G4/9, G5, G6/7, and G8. Genome Announc. 3, e01330-14 (2015).

Old Herborn University Seminar Monograph 30:
The magnificent microbiome - future aspects.
Editors: Peter J. Heidt, Thomas C.G. Bosch, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 115-132 (2017).

DEVELOPMENTAL EFFECTS OF EARLY LIFE EXPOSURE TO ENTERIC PATHOGENS AND OTHER ENVIRONMENTAL FACTORS

DENNIS R. LANG

Foundation for the National Institutes of Health, Bethesda, MD, USA, and
Fogarty International Center, NIH, Bethesda, MD, USA

INTRODUCTION

Almost fifty percent of the mortality of children under age five can be attributed to infectious diseases, with the majority of this global burden in Africa and South Asia (*Liu* et al., 2015). Additionally, childhood under-nutrition contributes to 30-40% of early childhood deaths and 70% of deaths due to diarrhoea (*UNICEF-WHO-World Bank Group*, 2015). Mortality rates have been declining over the past two decades due in part to the availability of vaccines against some respiratory and diarrhoeal pathogens, improved water quality and sanitation, the development and use of oral and intravenous rehydration solutions for dehydrating diarrhoea and an improvement in overall world economies. However, there are some areas of the world where these improvements have not been fully realised, and child mortality remains unacceptably high at rates three to four times those of developed countries (*Liu* et al., 2015).

Decline in the rates of morbidities associated with diarrhoeal disease have not been reduced to the same extent as those of mortality (*Kosek* et al., 2003). It has been recognized that children suffering from frequent episodes of diarrhoea may become malnourished as measured by stunted (length-for-age [LAZ] of ≤ -2), underweight (weight-for-age [WAZ] ≤ -2) or wasted (weight-for-length) [WLZ] ≤ -2) growth. Other longer-term morbidities such as reduced immune response to orally administered vaccines and impaired cognitive development and school performance have also been associated with the occurrence of diarrhoea in the first two years of life. The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) was initiated in 2008 to define the strength of association of enteric infection, undernutrition and other environmental exposures with the development of these longer-term morbidities. Such data are useful to better define the contribution of these exposures to the calculation of Disability Adjusted Life Years (DALY's) and in the hope that current and potential new interventions could be identified and prioritized.

MAL-ED is a prospective, longitudinal, observational birth cohort study. More than 200 new-borns were recruited from each of eight international sites with historically high rates of both diarrhoeal disease and stunted growth:

NOTE: This paper was originally presented at the 30th Old Herborn University Seminar in June 2016. Because some of the data presented at that meeting was unpublished, the print version of the presentation was embargoed until those data were published or accepted for publication in peer-reviewed journals. As of June of 2018, that has been accomplished. As a result, this manuscript has been updated so as to include that data and current literature citations.

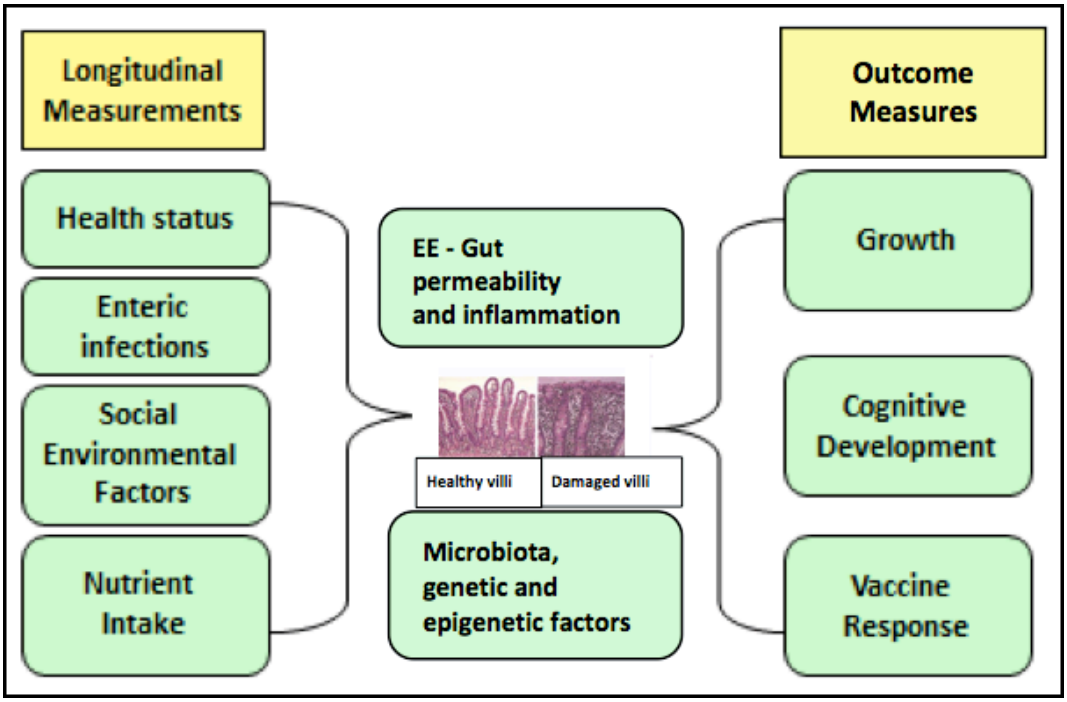

Figure 1: The MAL-ED hypotheses. Enteric infections were detected by examination of non-diarrhoeal surveillance and diarrhoeal stool throughout the study and were hypothesized to contribute to the development of Environmental Enteropathy (EE), characterized by measuring gut inflammation and increased permeability. A damaged gut was postulated to contribute to deficits in the major outcome measures of growth, cognitive development and immune response to routine scheduled childhood vaccines. Other variables including overall health status, social and environmental factors and nutrient intake were also considered as described previously (*MAL-ED Network Investigators*, 2014). This recognised the potential role for the gut microbiota and human genetic and epigenetic factors (which were not measured) that may also contribute to study outcomes.

four Asian sites located in Dhaka, Bangladesh (BGD); Vellore, India (INV); Bhaktapur, Nepal (NEB) and Nashero-Ferroze, Pakistan (PKN), two Southern African sites located in Venda, South Africa (SAV) and Haydom, Tanzania (TZH), and two South American sites located in Fortaleza, Brazil (BRF) and Loreto, Peru (PEL). MAL-ED collected information on the illness history of the cohort to include the incidence of diarrhoeal and respiratory disease identified through twice weekly home visits and caregiver reports. In contrast to other studies, we primarily identified cases of mild to moderate diarrhoeal disease that often go unrecorded due to lack of presentation to a health care facility**.** This study was also unique in that we collected monthly non-diarrhoeal stools in order to identify the enteric pathogens present in these samples as a way of assessing the overall rate of exposure of children.

The MAL-ED study hypothesised that frequent enteric infections (with or without diarrhoea) lead to the development of environmental enteropathy (EE), a condition characterized by intestinal inflammation, damaged gut architecture and decreased absorptive

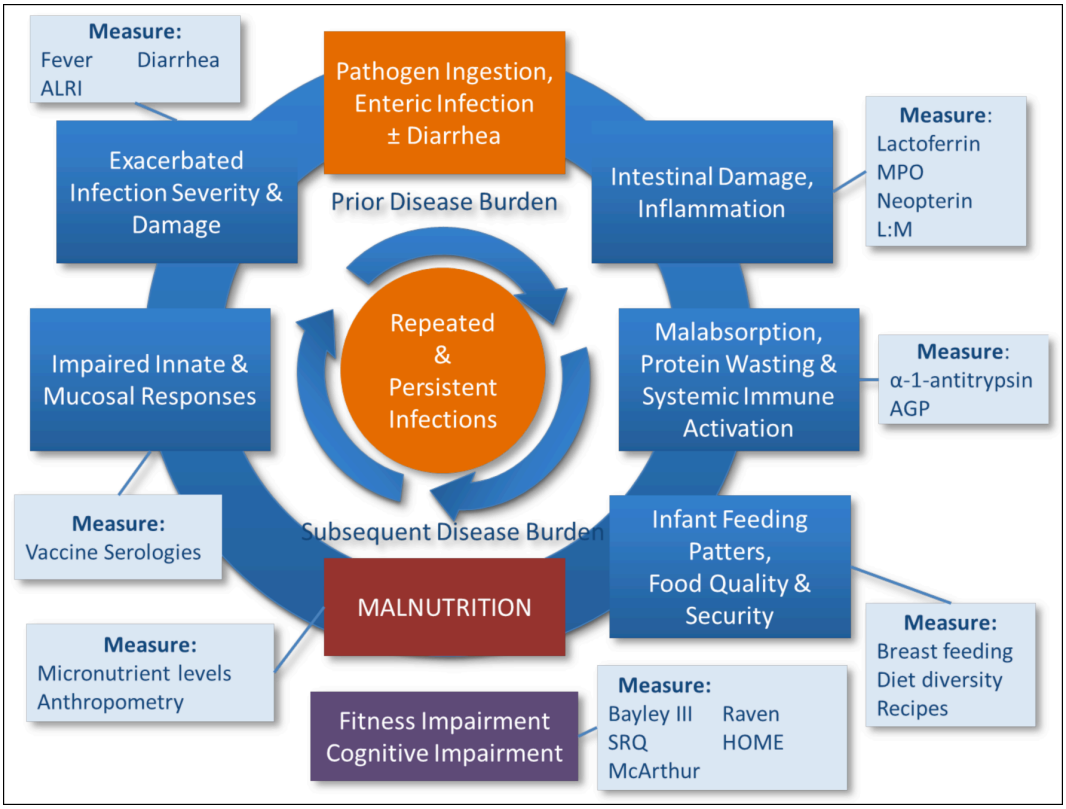

Figure 2: A depiction of the "vicious cycle of poverty" (*Guerrant* et al., 2008) of enteric infections leading to enteropathy and malabsorption of nutrients which, together with an inadequate diet, contributes to the development of malnutrition (growth deficits), cognitive impairment and decreased immune responses which, in turn, increase the child's susceptibility to more infections. The light blue boxes indicate some of the MAL-ED measures used to assess each step in this cycle.

capacity that was initially described histologically using gut biopsies. This condition, originally called tropical enteropathy, was first identified as altered intestinal architecture visualized in gut biopsies in individuals who travelled to or resided in areas that have high environmental burdens of enteropathogens and who lacked access to clean water and adequate sanitation (*Lindenbaum* et al., 1966; *Colwell* et al., 1968; *Fagundes-Neto* et al., 1984). MAL-ED further hypothesised that development of EE, in combination with an inadequate diet, leads to malnutrition manifested as growth deficits and the other morbidities as depicted in Figure 1. In children living in poverty, the progression of events from initial enteric infection to the development of enteropathy, undernutrition, impaired growth and cognitive development and suppressed immune response to certain vaccines has been proposed as a "vicious cycle of poverty" (*Guerrant* et al., 2008). Figure 2 depicts this cycle and indicates the data types that were collected throughout MAL-ED to evaluate the strength of evidence supporting this model.

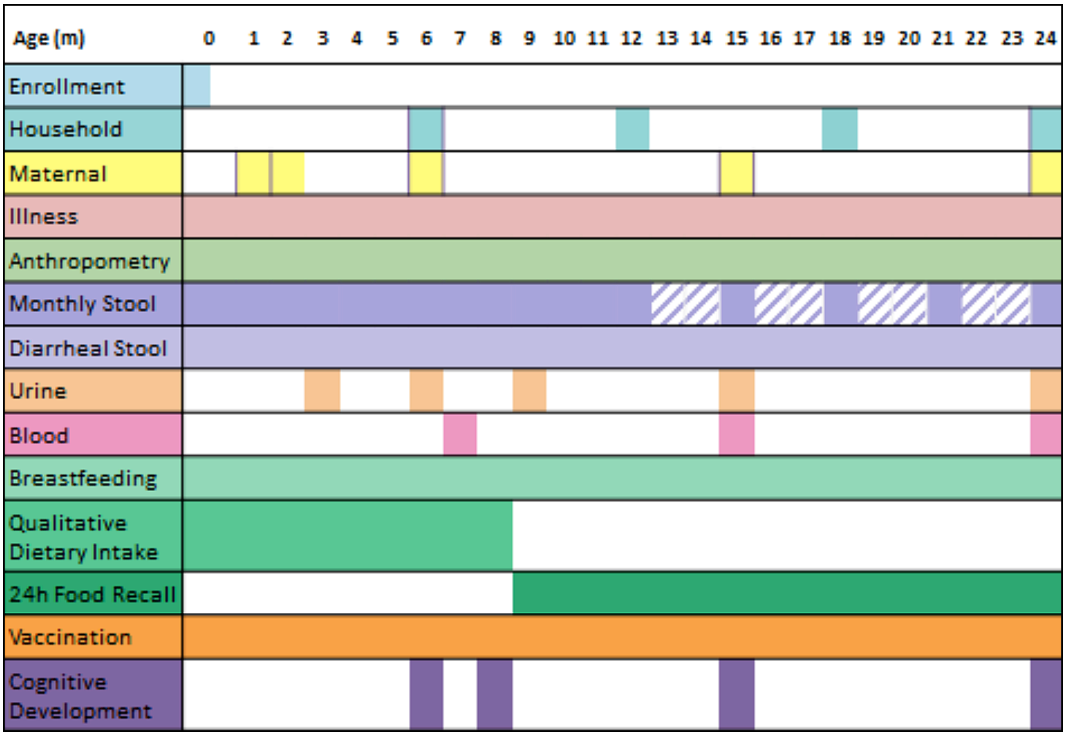

Figure 3: Data collection schedule. Colours refer to the different data types that were collected at the indicated months of age of the MAL-ED cohort children. For example, household assessments were done when the child was 6, 12, 18 and 24 months old and anthropometry measurements were schedule monthly for the duration of the study. The hashed months indicated as part of the monthly stool collections reflect samples that were collected but not analysed for presence of pathogens. These samples are maintained as study sample archives at the field sites where they were collected.

METHODS

Descriptions of the MAL-ED field sites, study design and methods have been previously described (*Lang*, 2010; *MAL-ED Network Investigators*, 2014) and are only briefly summarized here. Children were followed from enrolment near birth through the first 24 months of life. Variables of interest included enteropathogen exposure, illness history, dietary intakes and the home environment.

The protocol was approved by each local Institutional/Ethical Review Board (I/ERB) and by the IRBs at collaborating institutions in the United States, Norway and Thailand. Enrolment followed signed informed consent. In order to capture seasonal effects of exposures, monthly enrolment of between 15-18 new-borns was attempted at each site. The data collection schedule used in the study is shown in Figure 3. A data-coordinating centre (DCC) was established at the Fogarty International Center (NIH, Bethesda, MD, USA).

Biologic specimens are maintained at the site where they were collected and frozen at -80° C. All analyses were performed using de-identified data. Figure 4 illustrates the magnitude and breadth of some of the data types that were collected in the study.

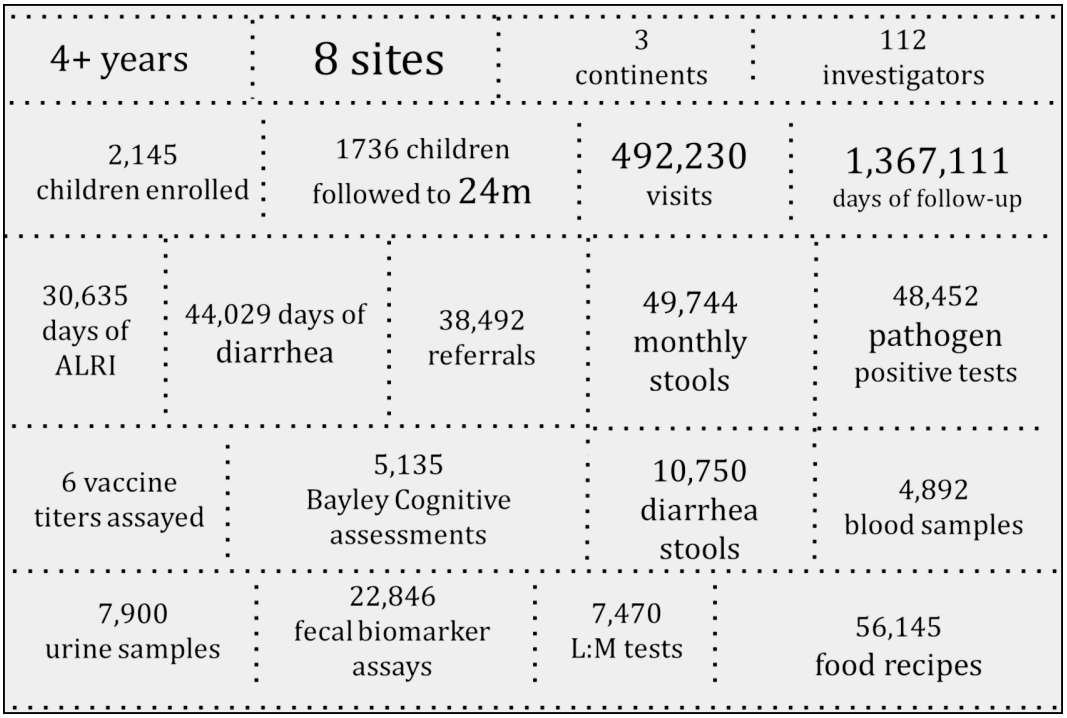

Figure 4: The magnitude and breadth of data types and samples collected within MAL-ED. This figure indicates the number and variety of a partial list of the data types that were collected during the study. Abbreviations used: ALRI = Acute Lower Respiratory Infection, L:M = Lactulose : Mannitol ratio.

Data were collected during twice weekly home visits by trained field workers who executed a common protocol that included physical measurement of children's length, weight and head circumference, and collection of stool samples, illness and dietary histories. A number of questionnaires were used to measure home environment, family socio-economic status (SES), a child's health, vaccination history, use of antibiotics and oral rehydration solution, extent of breastfeeding, weaning food composition, child temperament and the care-givers' education, parity, use of alcohol and tobacco products during pregnancy. Mother's reasoning ability was assessed by the Raven's Progressive Matrices (*Raven*, 2000).

Enteric pathogens were detected by standard microbiologic assays, microscopy, ELISA and molecular methods. EE biomarkers were determined by ELISA. As the study did not collect gut biopsies (the gold standard for assessing EE) from these very young children, we chose a panel of non-invasive biomarkers of inflammation (myeloperoxidase [MPO] and neopterin [NEO]) and gut permeability (alpha-1-antitrypsin [AAT]) assayed in stool. Urine samples were used to measure gut permeability by the lactulose : mannitol test and for some micronutrients. Plasma was used to measure vaccine immune response and systemic inflammation.

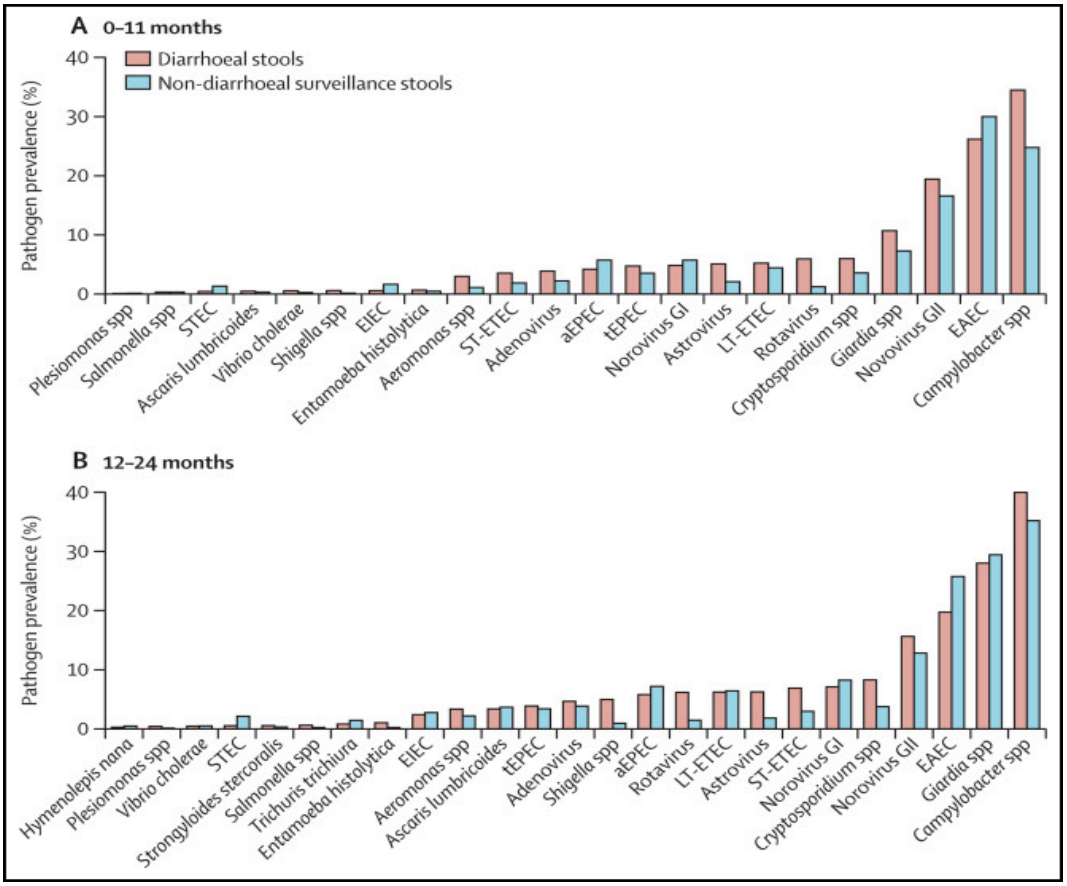

Figure 5: Pathogens detected in diarrhoeal (red bars) and non-diarrhoeal stools (blue bars), 0-11 months (A) and 12-24 months (B). EAEC=enteropathogenic *E. coli*; EIEC=enteroinvassive *E. coli*; aEPEC=atypical enteropathogenic *E. coli*; tEPEC=typical enteropathogenic *E. coli*; LT-ETEC=LT-producing enterotoxigenic *E. coli*; ST-ETEC=ST-producing enterotoxigenic *E. coli*; STEC=Shiga toxin-producing *E. coli*. Pathogens present in less than 0.1% of stool samples are not shown. Figure from *Platts-Mills* et al. (2015).

RESULTS

Enteric pathogen burden

We examined diarrhoeal stools (n = 7,318) and non-diarrhoeal surveillance stools collected monthly (n = 24,310) from cohort children for enteric pathogens (bacteria, viruses and parasites) as previously described (*Houpt* et al., 2014). The frequency of detection of the majority of these pathogens was higher in diarrhoeal than in non-diarrhoeal specimens (*Platts-Mills* et al., 2015). However, detection of some pathogens in non-diarrhoeal stools was higher, and for others nearly equivalent, to what was found in diarrhoeal stools during the first 24 months of life (Figure 5). The most frequently identified organisms in both diarrhoeal and non-diarrhoeal surveillance stools were *Campylobacter*, entero-aggregative *E. coli* (EAEC), *Giardia*, Norovirus and *Cryptosporidium* (*Platts-Mills* et al., 2015; Figure 5) although there was site-to-site heterogeneity in the rank order.

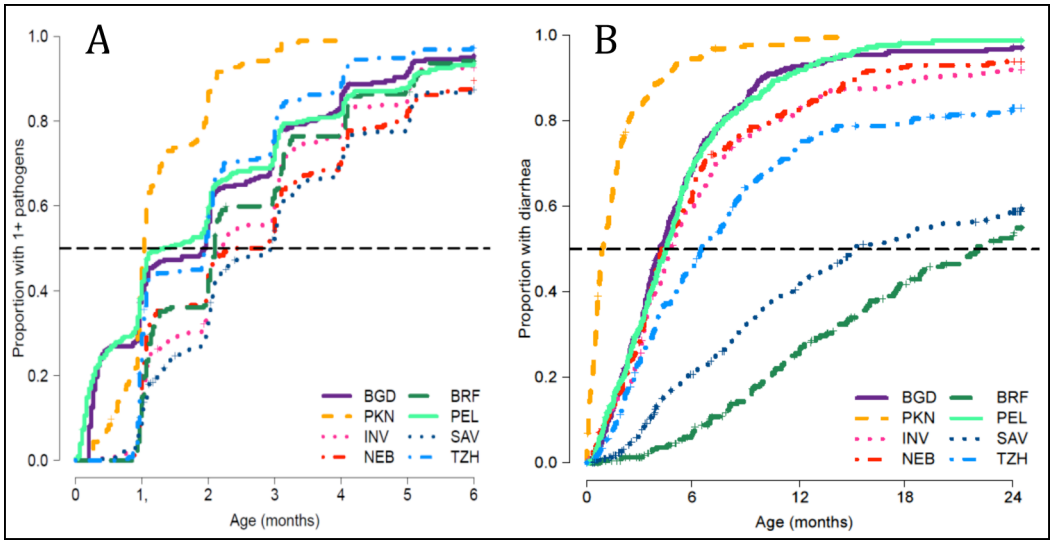

Figure 6: Proportion of children (vertical-axis) who have been infected with an enteric pathogen (A) and the proportion that have experienced their first episode of diarrhoea (B) during the first two years of life. The horizontal dotted line on both graphs indicates 50 % of children. Range of ages by which 50% of children have their first diarrhoea episode: 1 month in PKN, about 5-7 months in BGD, INV, PEL, NEB, and TZH, about 15 months in SAV and about 23 months in BRF.

Children experienced their first enteric infection early in life (Figure 6), with 50% of children at all sites infected by the time they were three months old and over 85% of these children had been infected at least once by the time they were six months of age, depending on the site. Despite this early high rate of infection, many children had not experienced diarrhoea until later in life, with the time to first diarrhoeal episode ranging from 1 month (PKN) to 22 months (BRF).

Several children at the BRF and SAV sites did not develop diarrhoea at all during their first two years of life, despite frequent enteric infections during that time. The extent of this enteric infectious burden is shown in Figure 7. Of note, is the percentage of diarrhoeal and non-diarrhoeal surveillance stools containing at least one pathogen that continued to increase throughout the study until between 80 and 100% of diarrhoeal stools and 70 to 90% of non-diarrhoeal surveillance stools contained at least one pathogen. Approximately 30-40% of all stool samples contain two or more pathogens (*Platts-Mills* et al, 2015*)* with as many as eight pathogens having been identified as co-infections in the same sample.

Growth of children

Between 10-20% of children at all sites were born stunted (<-2 LAZ), as illustrated in Figure 8. Only the BRF site observed a decline in the rate of stunting over the first two years where only 3.6% were stunted at 24 months of age. This is in contrast to the findings at other sites that exhibited an increased proportion of children stunted at two years of age, from between 23% in NEB to 70.6% in TZH. Slower linear growth was associated with the presence of enteric pathogens (cumulative burden of all measured pathogens) in non-diarrheal stool. Individual pathogens that had a negative association

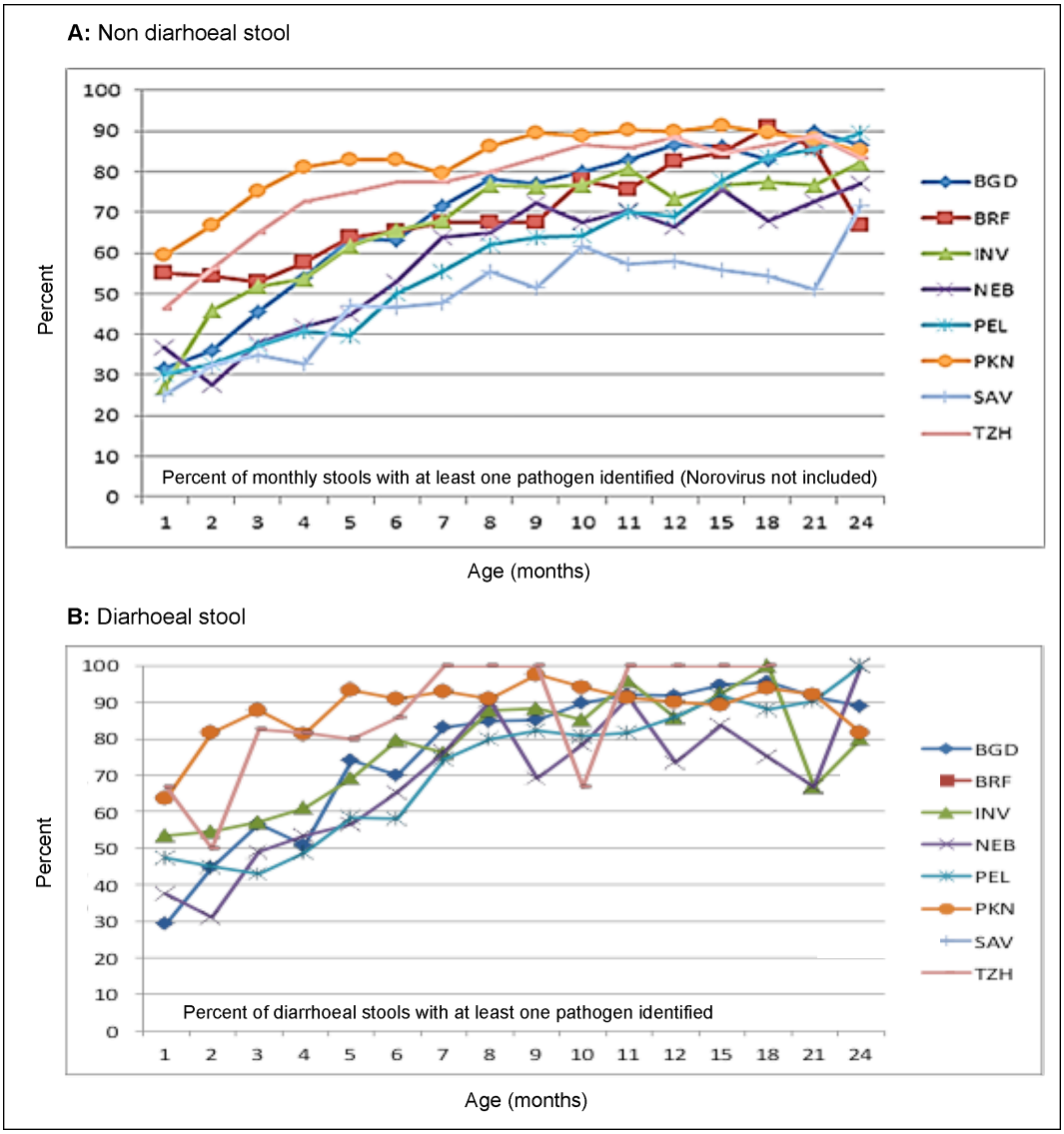

Figure 7: Percent of collected non-diarrhoeal surveillance (A) and diarrhoeal (B) stools containing at least one pathogen during the first two years of life, by site. Approximately 70-90% of all non-diarrhoeal surveillance stool samples collected from 8-24 month old children contain at least one enteric pathogen (bacteria, virus or parasite). Likewise, but perhaps more predictable, 80-100% of diarrhoeal specimens from 8-24 month old children also contained at least one enteric pathogen. Brazil and South Africa sites are not included in (b) due to their low frequency of diarrhoea.

were *Campylobacter* and enteroaggregative *E. coli.* Additionally, *Cryptosporidium*, LT-ETEC and atypical EPEC tended toward an association with slower growth rate. Diet also affected growth. Lower quality (lower energy and protein density) complementary diets from 9-24 months were associated with a slower growth rate and lower attained length at 24 months of age (*MAL-ED Investigators,* 2017a).

In a separate analysis of growth (*MAL-ED Network Investigators*, 2017b) we determined that maternal

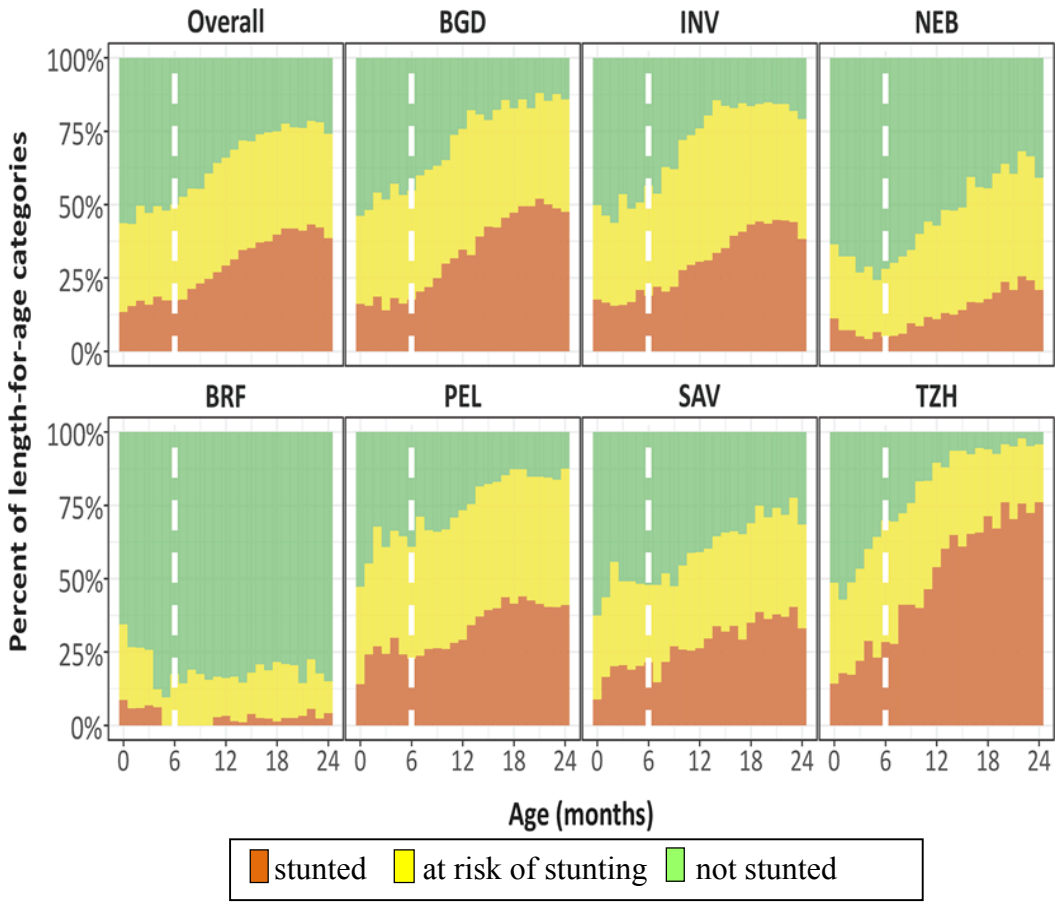

Figure 8. Categories of length-for-age stratified by exact month of age and site. In this figure, not stunted (length-for-age *Z*-score [LAZ] > −1) is represented in green, at risk of being stunted (−2 < LAZ < −1) is represented in yellow, and stunted (LAZ < −2) is represented in orange. Sites include BGD, Dhaka, Bangladesh; BRF, Fortaleza, Brazil; INV, Vellore, India; NEB, Bhaktapur, Nepal; PEL, Loreto, Peru; SAV, Venda, South Africa; TZH, Haydom, Tanzania. The vertical broken line represents 6 months of age. (Figure from *MAL-ED Network Investigators,* 2017b).

and prenatal factors were also important in early child growth. Maternal short stature and lower enrolment weight of the child were negatively associated with growth attained at 24 months of age. This analysis also identified enteropathogen burden in non-diarrheal stool, lower percent of energy from protein in the diet and lower household SES to be negatively associated with LAZ at 24 months. We have previously reported an association of elevated levels of the stool biomarkers of intestinal and systemic inflammation (MPO, MEO and AGP) and gut permeability with reduced growth (LAZ) over the six months following the biomarker measurements (*Kosek* et al., 2013, 2016).

As has been noted, reaching the stunting threshold is not the only measure that should be used to define suboptimal child growth (*Richard,* 2018). Even though there are many children in LMICs whose growth attainment does not reach that definition (< -2 LAZ), they are still likely to be growing at less than their full potential.

Cognitive development

Diarrhoea, enteric pathogens and subsequent malnutrition have been linked to impaired cognitive development (*Berkman* et al., 2002; *Patrick* et al., 2005; *Oria* et al., 2016). We examined the association of several environmental exposures with cognitive development as measured by the Bayley Scales of Infant and Toddler Development (BSID-III) (*Bayley*, 2005) at 24 months (*MAL-ED Network Investigators, 2018a)*. Factors that associated with lower BSID-III scores included illnesses indicators (vomiting, fever, and acute lower respiratory infection), higher frequencies of enteric pathogen detection both in diarrhoeal and non-diarrhoeal stools, and lower scores on the Raven's test of mothers reasoning ability. Illness and enteric pathogen burden effects were at least partially mediated through lower haemoglobin concentration. Factors associated with higher age-appropriate cognitive development scores included both physiologic factors such as higher levels of B vitamins (B6 and folate), haemoglobin, and social factors including a more nurturing home environment as measured by the Home Observation for the Measurement of the Environment (HOME) instrument (*Bradley* et al., 1996), and the mother's ability to form comparisons, reason by analogy, and organize spatial perceptions as assessed with the RCM.

Immune response to vaccines

Previous research has shown that enteric diseases and malnutrition also lead to reduced immune responses to certain childhood vaccines (*Myaux* et al., 1996; *Parker* et al., 2014). We assessed the immune responses to a number of childhood vaccines administered as part of the routine immunisation schedule in each country. These included two oral vaccines (oral polio in seven sites, oral rotavirus in three sites) as well as parenteral vaccines (measles, pertussis, and tetanus at all eight sites).

Our findings on factors effecting performance of the oral polio vaccine (OPV) have recently been published (*MAL-ED Network Investigators,* 2018b). Plasma was obtained and titres determined at 7 and 15 months of age. Failure to seroconvert to OPV administration was associated with high detection rates of enteric bacterial and parasitic pathogens in non-diarrhoeal surveillance stool samples during the neonatal period. This was especially true for poliovirus serotypes 2 and 3. Interestingly, viral pathogen burden did not exhibit similar negative effects. Biomarkers of gut function (increased L:M ratio and lower AAT) in stool were also weakly associated with poorer response to serotypes 2 and 3 of OPV. Factors associated with higher levels of seroconversion included receiving more and early vaccine doses and a higher SES, particularly having more household assets (an indicator of longer-term wealth) including improved water supply and improved sanitation in the home (Table 1).

DISCUSSION

The MAL-ED study examined the relative contributions of a number of environmental exposures to major outcomes of child development. It is unprecedented in the breadth of data types collected and the number of geographically dispersed sites studied using a common protocol. While many studies have examined the effect of diarrhoea on growth, cognitive development or immune response, MAL-ED was the first to intensively examine the

Table 1: Factors positively or negatively associated with the major study outcomes in MAL-ED children. Growth outcome was assessed as LAZ at 24 months of age, cognitive development was assessed with Bayley III at 24 months, and immune response to OPV (serotypes 1,2, and 3) were assessed at 7 and 15 months of age.

Outcome	Negative associations	Positive associations	References
Linear growth attainment at 24 months	1. Sub-clinical enteric infections 2. Infection with *Campylobacter* and EAEC (*Cryptosporidium*, LT-ETEC and aEPEC tended toward negative) 3. Elevated levels of gut inflammatory biomarkers in stool	1. Energy and protein from animal milk and dairy (3-8 months) 2. Protein from complimentary food (9-24 months) 3. Higher SES 4. Higher LAZ and WAZ at birth	*MAL-ED Investigators* (2016a,b) *Kosek* et al., (2013, 2016) *Rogawski* et al., (2017) *Amour* et al., (2016) *Korpe* et al., (2018)
Cognitive development at 24 months	1. Illness indicators (fever, vomiting, ALRI) 2. Enteropathogen detection in both diarrhoea and sub-clinical stool 3. Lower haemoglobin levels 4. Lower weight at enrolment	1. B vitamins (B6 and folate), higher haemoglobin 2. Home environment (HOME) 3. Mother's reasoning ability (RCM)	*MAL-ED Investigators* (2018a)
Immune response to oral polio vaccine (OPV)	1. Sub-clinical bacterial and parasitic infection during the neonatal period was a predictor of OPV failure, especially for serotypes 2 and 3. Viral infections did not exhibit similar relationships 2. Elevated L:M ratio and lower AAT were weakly associated with failure for serotypes 2 and 3 only.	1. Receiving more and early OPV doses improved response 2. Four doses maximized seroconversion of serotype 1 3. Early dosing and receiving five or more doses maximized response for serotype 3 4. Higher SES (household assets and improved water and sanitation) were the strongest predictors of seroconversion	*MAL-ED Investigators* (2018b)

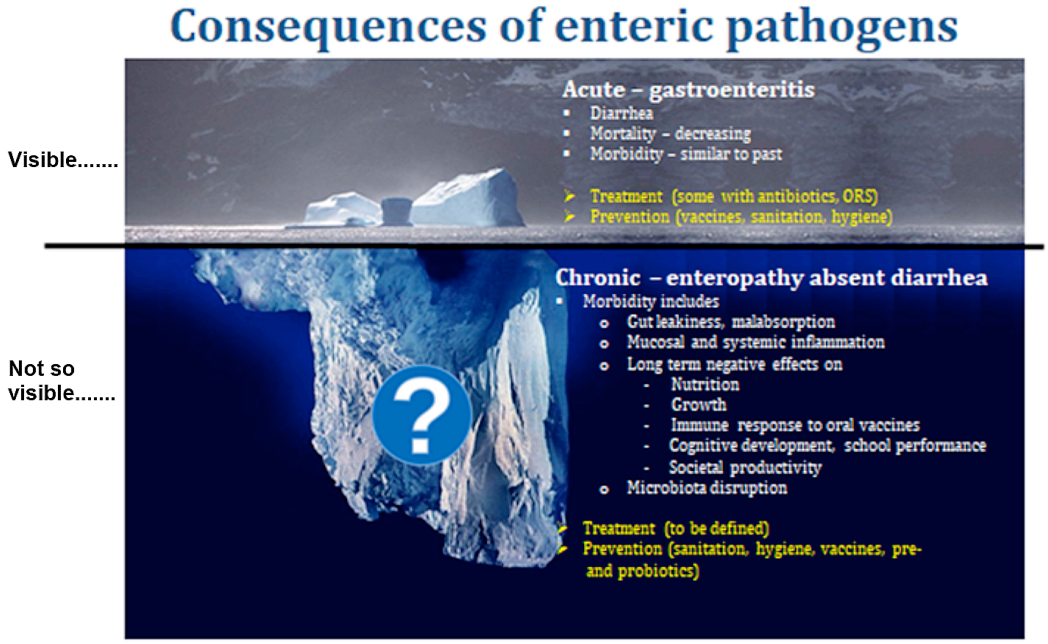

Figure 9: Depiction of both readily observed acute disease (diarrhoea and consequent mortality and morbidity) and the cryptic subclinical chronic effects (enteropathy, malnutrition, stunted growth, decreased cognitive development, impaired immune response, reduced school achievement and societal productivity) of enteric infections. The question mark highlights the currently unrecognized factors that could also contribute additional burden (e.g. new pathogens, dietary components, environmental xenobiotic chemicals, loss of beneficial commensal gut flora, and host genetic and epigenetic factors).

cumulative extent of enteric infections, both in diarrhoeal and non-diarrhoeal stools, within the context of multiple other environmental factors and their association with these developmental outcomes. We believe it was the first study to comprehensively characterize the longitudinal exposure of children, within a community setting, to enteric pathogens by recording their presence in non-diarrhoeal surveillance stool samples.

The observation that a high pathogen burden is negatively associated with all of the major child developmental outcomes - growth, cognitive development and immune response to vaccination - is, we believe, a critical outcome of this study. We have documented the extent to which children in these settings are infected with enteric pathogens; that these high infection rates persist over at least the first two years of life; and because most occur subclinically, largely go unrecognized. Therefore, they were not considered in calculations of the global burden of enteric diseases (*Global Burden of Disease Pediatrics Collaboration*, 2015).

It should be noted that in this study, we used classical microbiologic detection methods of culture, ELISA, microscopy. Our data implicate a number of bacterial and parasitic pathogens, though not viral pathogens, as having the most significant negative associations with the major study outcomes. When more sensitive and quantitative methods, e.g. quantitative-PCR (*Liu* et al., 2013) are used with MAL-ED samples (in progress) it is expected that

even more pathogens will be identified in both diarrhoeal and non-diarrhoeal samples as has been demonstrated in another study (*Liu* et al., 2016). Because this method is semi-quantitative, it may be possible to more accurately ascribe aetiology of diarrhoea or developmental deficits to particular pathogens.

MAL-ED data have revealed that the burden of subclinical (i.e. non-diarrhoeal) enteric infections on child morbidity is actually greater than that due to diarrhoea (Table 1 and Figure 9). A child with diarrhoea is easily recognized and treated. A child with few, if any, outward signs of morbidity but burdened none-the-less with enteric pathogens, is much more difficult to identify and to define pathologically. Clearly, validated biomarkers capable of detecting developing enteropathy are needed; markers that can be easily and inexpensively used to monitor very young infants in field studies. More work is needed to further evaluate the biomarkers used in MAL-ED and others (*Naylor* et al., 2015; *Guerrant* et al., 2016; *Kosek* et al., 2016) for their usefulness as indicators of clinically relevant pathology and for their potential as indicators of successful therapeutic intervention. Another biomarker of promise is a gut microbiota signature consisting of a limited number of microbial taxa found in children growing relatively well but lacking in children with severe malnutrition (*Subramanian* et al., 2014). If it were easy to identify the strength of this "signature" profile in stool samples collected longitudinally, it might allow the identification of at risk children and the ability to monitor improvement following intervention.

With any biomarker, it would be helpful to have ranges of values obtained from comparably aged children in developed countries. Such data would help in the interpretation of the values observed in studies in LMICs. However, the use of periodic child length measurements may remain the cheapest, quickest and most reproducible method to detect at-risk children and to monitor the success of any intervention.

We have identified the early exposure to and asymptomatic carriage of enteropathogens and dietary deficiencies as important contributors to less than optimal growth in this cohort of children (*MAL-ED Network Investigators, 2017a, 2017b)*. Reduction in the exposure to enteric pathogens could reduce the level of growth faltering in young children. When combined with efforts to improve the quality, quantity and diversity of complementary food may yield improvement in growth velocity during the critical first two years of life. Our observations that low maternal height, low neonatal weight and low SES also have deleterious effects on child growth attainment throughout the first 24 months suggest that earlier pre-natal interventions could be helpful (*MAL-ED Network Investigators*, 2017B). The observed negative association of asymptomatic infections extended to the other long-term outcomes of cognitive development (*MAL-ED Network Investigators,* 2018a*)* and OPV immune response (*MAL-ED Network Investigators,* 2018b*)* (also see Table 1), emphasizes the importance of reducing environmental exposure to these pathogens, improved nutrition and higher SES as predictable effectors of improved child development.

Despite our efforts to account for many of the factors contributing to child development shortfalls, substantial inter-site variability remained unaccounted for. As indicated by the question mark in Figure 9, we acknowledge that there are unknown factors that also

may contribute to developmental shortfalls that are likely to occur in different quantitative combinations in different locations. Among these factors could be new, as yet unidentified enteric pathogens, environmental xenobiotic chemical contaminants, dietary components or toxins, genetic and epigenetic factors and the absence or imbalance of certain gut microbes.

The fact that impaired development is negatively associated with multiple factors, suggests that a combination of interventions, applied in concert, may be needed in order to see significant improvement, improvements typically found when public health improves and poverty decreases. A first step is recognizing that early exposure to enteric pathogens is common in these settings, and that the ensuing repeated subclinical infections during the first few months and years of life is an important contributor to the subsequent delayed development of children.

ACKNOWLEDGEMENTS

The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) is carried out as a collaborative project supported by the Bill & Melinda Gates Foundation, the Foundation for the NIH, and the National Institutes of Health, Fogarty International Center. The author thanks the staff and participants of the MAL-ED Network for their important contributions. Helpful editorial suggestions on the manuscript were provided by Drs. Ben McCormick, Michael Gottlieb and Karen Tountas, for which I am grateful.

LITERATURE

Amour, C., Gratz, J., Mduma, E., Svensen, E, Rogawski, E.T., McGrath, M., Seidman, J.C., McCormick, B.J., Shrestha, S., Samie, A., Mahfuz, M., Qureshi, S., Hotwani, A., Babji, S., Trigoso, D.R., Lima, A.A., Bodhidatta, L., Bessong, P., Ahmed, T., Shakoor, S., Kang, G., Kosek, M., Guerrant, R.L., Lang, D., Gottlieb, M., Houpt, E.R., Platts-Mills, J.A.; Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) Network Investigators: Epidemiology and Impact of Campylobacter Infection in Children in 8 Low-Resource Settings: Results from the MAL-ED Study. Clin. Infect. Dis. 63,1171-1179 (2016).

Bayley, N.: Bayley scales of infant and toddler development, Third Edition (Bayley-III®). Harcourt Assessment, San Antonio, TX, USA (2005).

Berkman, D.S., Lescano, A.G., Gilman, R.H., Lopez, S.L., and Black, M.M.: Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet 359, 564-571 (2002).

Bradley, R.H., Corwyn, R.F., and Whiteside-Mansell, L.: Life at home: Same time, different places — An examination of the HOME Inventory in different cultures. Early Dev. Parent. 5, 251–269 (1996).

Colwell, E.J., Welsh, J.D., Legters, L.J., and Proctor, R.F.: Jejunal morphological characteristics in South Vietnamese residents: A preliminary report. JAMA 206, 2273-2276 (1968).

Fagundes-Neto, U., Viaro, T., Wehba, J., da Silva Patricio, F.R., and Machado, N.L.: Tropical enteropathy (Environmental Enteropathy) in early childhood: a syndrome caused by contaminated environment. J.

Trop. Pediatr. 30, 204-209 (1984).

Global Burden of Disease Pediatrics Collaboration: Kyu, H.H., Pinho, C., Wagner, J.A., Brown, J.C., Bertozzi-Villa, A., Charlson, F.J., Coffeng, L.E., Dandona, L., Erskine, H.E., Ferrari, A.J., Fitzmaurice, C., Fleming, T.D., Forouzanfar, M.H., Graetz, N., Guinovart, C., Haagsma, J., Higashi, H., Kassebaum, N.J., Larson, H.J., Lim, S.S., Mokdad, A.H., Moradi-Lakeh, M., Odell, S.V., Roth, G.A., Serina, P.T., Stanaway, J.D., Misganaw, A., Whiteford, H.A., Wolock, T.M., Wulf Hanson, S., Abd-Allah, F., Abera, S.F., Abu-Raddad, L.J., AlBuhairan, F.S., Amare, A.T., Antonio, C.A., Artaman, A., Barker-Collo, S.L., Barrero, L.H., Benjet, C., Bensenor, I.M., Bhutta, Z.A., Bikbov, B., Brazinova, A., Campos-Nonato, I., Castañeda-Orjuela, C.A., Catalá-López, F., Chowdhury, R., Cooper, C., Crump, J.A., Dandona, R., Degenhardt, L., Dellavalle, R.P., Dharmaratne, S.D., Faraon, E.J., Feigin, V.L., Fürst, T., Geleijnse, J.M., Gessner, B.D., Gibney, K.B., Goto, A., Gunnell, D., Hankey, G.J., Hay, R.J., Hornberger, J.C., Hosgood, H.D., Hu, G., Jacobsen, K.H., Jayaraman, S.P., Jeemon, P., Jonas, J.B., Karch, A., Kim, D., Kim, S., Kokubo, Y., Kuate Defo, B., Kucuk Bicer, B., Kumar, G.A., Larsson, A., Leasher, J.L., Leung, R., Li, Y., Lipshultz, S.E., Lopez, A.D., Lotufo, P.A., Lunevicius, R., Lyons, R.A., Majdan, M., Malekzadeh, R., Mashal, T., Mason-Jones, A.J., Melaku, Y.A., Memish, Z.A., Mendoza, W., Miller, T.R., Mock, C.N., Murray, J., Nolte, S., Oh, I.H., Olusanya, B.O., Ortblad, K.F., Park, E.K., Paternina Caicedo, A.J., Patten, S.B., Patton, G.C., Pereira, D.M., Perico, N., Piel, F.B., Polinder, S., Popova, S., Pourmalek, F., Quistberg, D.A., Remuzzi, G., Rodriguez, A., Rojas-Rueda, D., Rothenbacher, D., Rothstein, D.H., Sanabria, J., Santos, I.S., Schwebel, D.C., Sepanlou, S.G., Shaheen, A., Shiri, R., Shiue, I., Skirbekk, V., Sliwa, K., Sreeramareddy, C.T., Stein, D.J., Steiner, T.J., Stovner, L.J., Sykes, B.L., Tabb, K.M., Terkawi, A.S., Thomson, A.J., Thorne-Lyman, A.L., Towbin, J.A., Ukwaja, K.N., Vasankari, T., Venketasubramanian, N., Vlassov, V.V., Vollset, S.E., Weiderpass, E., Weintraub, R.G., Werdecker, A., Wilkinson, J.D., Woldeyohannes, S.M., Wolfe, C.D., Yano, Y., Yip, P., Yonemoto, N., Yoon, S.J., Younis, M.Z., Yu, C., El Sayed Zaki, M., Naghavi, M., Murray, C.J., and Vos, T.: Global and national burden of diseases and injuries among children and adolescents between 1990 and 2013: Findings from the Global Burden of Disease 2013 study. JAMA Pediatrics 170, 267-287 (2015).

Guerrant, R.L., Oriá, R.B., Moore, S.R., Oriá, M.O., and Lima, A.A.: Malnutrition as an enteric infectious disease with long-term effects on child development. Nutr. Rev. 66, 487-505 (2008).

Guerrant, R.L., Leite, A.M., Pinkerton, R., Medeiros, P.H., Cavalcante, P.A., DeBoer, M., Kosek, M., Duggan, C., Gewirtz, A., Kagan, J.C., Gauthier, A.E., Swann, J., Mayneris-Perxachs, J., Bolick, D.T., Maier, E.A., Guedes, M.M., Moore, S.R., Petri, W.A., Havt, A., Lima, I.F., Prata, M.M., Michaleckyj, J.C., Scharf, R.J., Sturgeon, C., Fasano, A., and Lima, A.A.: Biomarkers of environmental enteropathy, inflammation, stunting, and impaired growth in children in Northeast Brazil. PLos One 11, e0158772 (2016).

Houpt, E., Gratz, J., Kosek, M., Zaidi, A.K., Qureshi, S., Kang, G., Babji, S., Mason, C., Bodhidatta, L., Samie, A., Bessong, P., Barrett, L., Lima, A., Havt, A., Haque, R., Mondal, D., Taniuchi, M., Stroup, S., McGrath, M., Lang, D.; MAL-ED Network Investigators: Microbiologic methods utilized in the MAL-ED cohort study. Clin. Infect. Dis. 59, Suppl. 4, S225-232 (2014).

Kosek, M., Bern, C., and Guerrant, R.L.: The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. World Health Organ. 81, 197-204 (2003).

Kosek, M., Haque, R., Lima, A., Babji, S., Shrestha, S., Qureshi, S., Amidou, S., Mduma, E., Lee, G., Yori, P.P., Guerrant,

R.L., Bhutta, Z., Mason, C., Kang, G., Kabir, M., Amour, C., Bessong, P., Turab, A., Seidman, J., Olortegui, M.P., Quetz, J., Lang, D., Gratz, J., Miller, M., Gottlieb, M.; MAL-ED network: Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants. Am. J. Trop. Med. Hyg. 88, 390-396 (2013).

Kosek, M.N., Mduma, E., Mduma, E., Kosek, P.S., Lee, G.O., Svensen, E., Pan, W.K., Olortegui, M.P., Bream, J.H., Patil, C., Asayag, C.R., Sanchez, G.M., Caulfield, L.E., Gratz, J., Yori, P.P.: Plasma Tryptophan and the Kynurenine–Tryptophan Ratio are Associated with the Acquisition of Statural Growth Deficits and Oral Vaccine Underperformance in Populations with Environmental Enteropathy. Am. J. Trop. Med. Hyg. 95, 928-937 (2016).

Kotloff, K.L., Blackwelder, W.C., Nasrin, D., Nataro, J.P., Farag, T.H., van Eijk, A., Adegbola, R.A, Alonso, P.L., Breiman, R.F., Faruque, A.S., Saha, D., Sow, S.O., Sur, D., Zaidi, A.K., Biswas, K., Panchalingam, S., Clemens, J.D., Cohen, D., Glass, R.I., Mintz, E.D., Sommerfelt, H., and Levine, M.M.: The Global Enteric Multicenter Study (GEMS) of diarrheal disease in infants and young children in developing countries: epidemiologic and clinical methods of the case/control study. Clin. Infect. Dis. 55, Suppl. 4, S232-S245 (2012).

Lang, D. The MAL-ED Project: Deciphering the relationships among normal gut flora, enteric infection and malnutrition and their association with immune response to vaccines. In: Development of strategies to overcome barriers to effective mucosal immunization of infants in developing countries. Old Herborn University Monograph 24 (Eds.: Heidt, P.J., Walker, R.I., and Rusch, V.). Old Herborn University Foundation, Herborn-Dill, Germany, 73-82 (2010): http://www.old-herborn-university.de/wp-content/uploads/publications/books/OHUni_book_24_article_7.pdf

Lindenbaum, J., Alam, A.K., and Kent, T.H.: Subclinical small-intestinal disease in East Pakistan. Br. Med. J. 2, 1616-1619 (1966).

Liu, J., Gratz, J., Amour, C., Kibiki, G., Becker, S., Janaki, L., Verweij, J.J., Taniuchi, M., Sobuz, S.U., Haque, R., Haverstick, D.M., and Houpt, E.R.: A Laboratory-Developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. J. Clin. Microbiol. 51, 472-480 (2013).

Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J.E., Cousens, S., Mathers, C., and Black, R.E.: Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet 385, 430-440 (2015).

Liu, L, Platts-Mills, J.A., Juma, J. et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. Lancet 388, 1291-3001 (2016).

MAL-ED Network Investigators.: Acosta, A.M., Chavez, C.B., Flores, J.T., Olotegui, M.P., Pinedo, S.R., Trigoso, D.R., Vasquez, A.O., Ahmed, I., Alam, D., Ali, A., Bhutta, Z.A., Qureshi, S., Shakoor', S., Soofi, S., Turab, A., Yousafzai, A.K., Zaidi, A.K., Bodhidatta, L., Mason, C.J., Babji, S., Bose, A., John, S., Kang, G., Kurien, B., Muliyil, J., Raghava, M.V., Ramachandran, A., Rose, A., Pan, W., Ambikapathi, R., Carreon, D., Charu, V., Dabo, L., Doan, V., Graham, J., Hoest, C., Knobler, S., Lang, D., McCormick, B., McGrath, M., Miller, M., Mohale, A., Nayyar, G., Psaki, S., Rasmussen, Z., Richard, S., Seidman, J., Wang, V., Blank, R., Gottlieb, M., Tountas, K., Amour, C., Mduma, E., Ahmed, T., Ahmed, A.M., Dinesh, M., Tofail, F., Haque, R., Hossain, I., Islam, M., Mahfuz, M., Chandyo, R.K., Shrestha, P.S., Shrestha, R., Ulak, M., Black, R., Caulfield, L., Checkley, W., Chen, P., Kosek, M., Lee, G., Yori, P.P., Murray-Kolb, L., Schaefer, B., Pendergast, L., Abreu, C., Bindá, A., Costa, H., Di Moura, A., Filho, J.Q., Leite, A., Lima, A., Lima, N., Lima, I., Maciel, B., Moraes, M., Mota, F., Oria, R., Quetz,

J., Soares, A., Svensen, E., Tor, S., Patil, C., Bessong, P., Mahopo, C., Mapula, A., Nesamvuni, C., Nyathi, E., Samie, A., Barrett, L., Gratz, J., Guerrant, R., Houpt, E., Olmsted, L., Petri, W., Platts-Mills, J., Scharf, R., Shrestha, B., and Shrestha, S.K.: The MAL-ED Study: A Multinational and Multidisciplinary Approach to Understand the Relationship Between Enteric Pathogens, Malnutrition, Gut Physiology, Physical Growth, Cognitive Development, and Immune Responses in Infants and Children Up to 2 Years of Age in Resource-Poor Environments. Clin. Infect. Dis. 59, Suppl. 4, S193–S206 (2014).

MAL-ED Network Investigators: Relationship between growth and illness, enteropathogens, and dietary intakes in the first 2 years of life: findings from the MAL-ED birth cohort study. BMJ Glob. Health 2, e000370 (2017a)

MAL-ED Network Investigators: Childhood stunting in relation to the pre- and postnatal environment during the first 2 years of life: The MAL-ED longitudinal birth cohort study. PLoS Med. 14, e1002408 (2017b).

MAL-ED Network Investigators.: Early childhood cognitive development is affected by interactions among illness, diet, enteropathogens and the home environment: findings from the MAL-ED birth cohort study. Br. Med. J., In press (2018a)

MAL-ED Network Investigators: Oral polio vaccine response in the MAL-ED birth cohort study: Considerations for polio eradication strategies. Vaccine, In press (2018b)

Myaux, J.A., Unicomb, L., Besser, R.E., Modlin, J.F., Uzma, A., Islam, A.M., and Santosham, M.: Effect of diarrhea on the humoral response to oral polio vaccination. Pediatr. Infect. Dis. J. 15, 204-209 (1996).

Naylor, C., Lu, M., Haque, R., Mondal, D., Buonomo, E., Nayak, U., Mychaleckyj, J.C., Kirkpatrick, B., Colgate, R., Carmolli, M., Dickson, D., van der Klis, F., Weldon, W., Steven Oberste, M.; PROVIDE study teams, Ma, J.Z., Petri, W.A. Jr.: Environmental Enteropathy, Oral Vaccine Failure and Growth Faltering in Infants in Bangladesh. EBioMedicine 2, 1759-1766 (2015).

Oria, R.B., Murray-Kolb, L.E., Scharf, R.J., Pendergast, L.L., Lang, D.R., Kolling, G.L., and Guerrant, R.L.: Early-life enteric infections: relation between chronic systemic inflammation and poor cognition in children. Nutr. Rev. 74, 374-386 (2016).

Parker, E.P.K., Kampmann, B., Kang, G., and Grassly, N.C.: Influence of enteric infections on response to oral poliovirus vaccine: a systematic review and meta-analysis. J. Infect. Dis. 210, 853-864 (2014).

Patrick, P.D., Oriá, R.B., Madhavan, V., Pinkerton, R.C., Lorntz, B., Lima, A.A., and Guerrant, R.L.:. Limitations in verbal fluency following heavy burdens of early childhood diarrhea in Brazilian shantytown children. Child. Neuropsychol. 11, 233-244 (2005).

Platts-Mills, J.A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., McCormick, B.J., McGrath, M., Olortegui, M.P., Samie, A., Shakoor, S., Mondal, D., Lima, I.F., Hariraju, D., Rayamajhi, B.B., Qureshi, S., Kabir, F., Yori, P.P., Mufamadi, B., Amour, C., Carreon, J.D., Richard, S.A., Lang, D., Bessong, P., Mduma, E., Ahmed, T., Lima, A.A., Mason, C.J., Zaidi, A.K., Bhutta, Z.A., Kosek, M., Guerrant, R.L., Gottlieb, M., Miller, M., Kang, G., Houpt, E.R.; MAL-ED Network Investigators: Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob. Health 3, e564-e575 (2015).

Raven, J.: The Raven's Progressive Matrices: change and stability over culture and time. Cogn. Psychol. 41, 1–48 (2000).

Richard SA, McCormick BJJ, Seidman JC, Rasmussen, Z., Kosek, M.N., Rogawski, E.T., Petri, W., Bose, A., Mduma, E., Maciel, B.L.L., Chandyo, R.K., Bhutta, Z., Turab, A., Bessong, P., Mahfuz, M., Caulfield, L.E., On Behalf Of The Mal-Ed Network Investigators: Relationships among Common Illness Symptoms and the Protective Effect of Breastfeeding in Early Childhood in MAL-ED: An Eight-Country Cohort Study. Am. J. Trop. Med. Hyg. 98,

904-912 (2018).
Rogawski, E.T., Guerrant, R.L., Havt, A., Lima, I.F.N., Medeiros, P.H.Q.S., Seidman, J.C., McCormick, B.J.J., Babji, S., Hariraju, D., Bodhidatta, L., Shrestha, J., Anania, J., Maro, A., Samie, A., Yori, P.P., Qureshi, S., Mahfuz, M., Bessong, P.O., Kosek, M.N., Ahmed, T., Bhutta, Z.A., Lang, D.R., Gottlieb, M., Houpt, E.R., Lima, A.A.M.; MAL-ED Network Investigators: Epidemiology of enteroaggregative Escherichia coli infections and associated outcomes in the MAL-ED birth cohort. PLoS Negl. Trop. Dis. 11, e0005798 (2017).
Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., Benezra, A., DeStefano, J., Meier, M.F., Muegge, B.D., Barratt, M.J., VanArendonk, L.G., Zhang, Q., Province, M.A., Petri, W.A. Jr., Ahmed, T., and Gordon, J.I.: Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature 510,417-421 (2014).
UNICEF-WHO-World Bank Group: Joint child malnutrition estimates. Key findings of the 2015 edition (2015): http://www.who.int/nutrition/publications/jointchildmalnutrition_2015_estimates/en/