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GENERAL RULES FOR ESTABLISHMENT AND MAINTENANCE OF GASTROINTESTINAL ECOSYSTEMS

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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.***

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