(blue baby syndrome) and cancer (via formation of nitrosamines). Over the years, the fear of nitrate flourished, despite lack of evidence for a carcinogenic effect in humans. Recent research has found that nitrate and its metabolite nitrite may possess some beneficial properties (14,15) and it seems as we a facing somewhat of a paradigm shift.

Comparative studies in germfree and conventional animals have clearly shown that the gastrointestinal microbiota play a substantial role in the nitrogen metabolism of the host (16,17,18). When ingested, nitrate is rapidly absorbed and actively excreted into saliva. Oral microorganisms will rapidly convert nitrate to nitrite and further to nitric oxide, both compounds are known to inhibit some bacteria and yeast – and to be utilized by others. If reaching the stomach, nitrite is non-enzymatically converted to nitric oxide under acidic conditions. It is well known that mammals produce nitric oxide in various cells, but the substrate is arginine and not nitrate. Nitric oxide is one of the most reactive molecule to be found in the mammalian body and is accepted to be a biological messenger of key importance. Obviously, the microorganisms in the digestive tract play a major role in nitrate metabolism. The difference in nitric oxide concentration in the stomach of conventional rats are significantly higher (up to 3 logs difference) in conventional than in germfree rats (17), Additionally, intestinal concentration of nitric oxide can be increased by giving nitrite to the animal (17). Some probiotic bacteria can convert nitrite to nitric oxide whereas other bacteria., as staphylocci, may be able to utilize formed nitric oxide. The biological significance of the microbial influence on ingested nitrate and nitrite is not fully understood. The key point is that some microbes convert nitrate to a compound that may act upon the host as well as upon other parts of the microbiotia.

Creatinine is usually regarded as an end product since no mechanism is known whereby creatinine can be degraded in mammalian tissue and it is excreted to feces and urine. However, it is puzzling that methylguanidine, a metabolite deriving from breakdown of creatinine is found in urine of germfree rats (13). If this old observation can be substantiated, it might be wise taking a new look on creatinine metabolism.; i.e. it might not be an end product.

Conclusion: The main lesson from several comparative studies in germfree and conventional animals is the gastrointestinal microbiota is receiving nitrogen from the host, but the net outcome is that the microorganism are helping the host in saving nitrogen. Thus, host-microbial interactions shape the nutrient environment of the mammalian gastrointestinal tract. It is a win-win situation for both parts. Germfree animals need substantial more nitrogen in their diet than their conventional counterparts.

Given the same amount of food, ex-germfree animals will gain weight when they are conventionalized. Alterations in recycling of nitrogen might account for some of this increase in weight.

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Lecture 2.1.3

Influence of the microflora on gastrointestinal nitric oxide (NO) generation: studies in newborn infants and germ-free animals

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The intestinal flora can elicit a number of effects in the gut, however the mechanisms behind this are not entirely understood (1). The communication between bacteria and the host occurs via receptors and through the secretion of chemical mediators. The studies presented here suggest that several common intestinal bacteria can generate nitric oxide (NO), a potent biological messenger with a variety of known physiological functions. NO controls almost a limitless range of functions in the body and is well accepted as biological messenger of key importance, involved in the regulation of regional blood flow, respiration, gut motility, water and electrolyte transport and immunity (2). In mammalian cells NO is synthesized from the amino acid L-arginine and molecular oxygen by nitric oxide synthases (NOS). NO is also

formed in the human body by reduction of nitrite (NO_2- →NO), as first described in the stomach. Denitrifying bacteria in soil generate NO from nitrate (NO_3-) and nitrite (NO_2-) as a part of the nitrogen cycle and the impact of bacteria and nitrite on human health has been suggested (3). The main focus of the studies described below has been to investigate whether the micro-organisms residing in the gastrointestinal (GI) tract could contribute to endogenous NO generation and under which conditions this would occur *in vivo*.

We developed several new unique methods to directly measure gaseous NO *in vivo* in the colon of newborn infants and in the entire GI tract of conventional and germ-free animals. In addition, we investigated NO generation and consumption by different gut bacteria by measuring gaseous NO under aerobic and anaerobic conditions.

First we monitored the initial bacterial colonization in newborn infants born vaginally or via Caesarian secton following the intracolonic hydrogen gas (H_2), faecal short-chain fatty acids (SCFA) and NO (4). All these markers were virtually undetectable at birth but increased in a particular pattern-bacterial products (H_2 and SCFA) appeared first followed by NO some days later. Interestingly, in some apparently healthy infants, colonic NO levels increased to levels similar to those seen in adults with inflammatory bowel disease, indicating a vivid activation of the immune system in response to the emerging bacterial flora.

Since the infant gut is exposed to a myriad of colonizing bacteria directly after birth5, we speculated that bacteria themselves could contribute to the colonic NO generation in addition to the mucosa. Our experiments showed that in conventional rats, NO levels were distinctly compartmentalized with very high levels in the stomach, intermediate levels in the caecum and lower levels in the small intestine and colon. In contrast, in germ-free rats, NO gas was low throughout the gastrointestinal tract. When we fed rats nitrate, gastric NO increased greatly in conventional but not in germ-free animals, thereby confirming nitrate to be a substrate for bacterial NO generation (6).

The effects and mechanisms of action of probiotics are far from being understood (7). We suggested and went on to demonstrate that lactic acid producing bacteria can generate considerable amounts of NO from nitrite *in vitro* (8). Reduction of nitrite to NO was likely non-enzymatic, caused by bacterial generation of acid. In the same study we measured *in vitro* gaseous NO generation from human faeces. Mixed faecal flora generated NO not only from nitrite but also from nitrate without a concomitant drop in pH, which suggested pathways other than acidification of nitrite. We demonstrated that intestinal NO generation can be stimulated in rats by dietary supplementation with nitrate and lactobacilli. Furthermore, in vitro studies showed that the generation of NO by some probiotic bacteria might be counteracted by rapid NO consumption by other strains (*E. coli* and *S. aureus*). We also studied if nitrite/nitrate in breast milk would interact with NO generation during the first days after birth and found a correlation between the colonic NO levels and nitrite in breast milk and in faeces of newborns (9).

Does luminal NO originates from the mucosa or is generated by bacteria? This information might be important, since an induced mucosal NO synthesis by NOS's would signal a possible inflammatory reaction. It seems to be equally important to know if NO can be generated by bacteria themselves as this could have a physiological impact on the host. In principle, there are two ways in which bacteria could contribute to intestinal NO production: bacteria stimulate cells in the mucosa to produce NO and that bacteria produce NO themselves. Judging from our studies, both mechanisms are possible but they seem to operate at different locations.

The newborn infant seem to be exposed to considerable levels of nitrite and nitrate during the first weeks of life as the physiological levels in the breast milk are highest immediately after birth and progressively decline with time. The establishing micro-flora may directly utilize the breast milk nitrate, converting it from an inert stable anion that the human cells cannot use into the more reactive nitrite. This nitrite could be toxic, but if further converted into nitric oxide, it might have possible beneficial physiological effects (10). In this way, increased amounts of nitrate in breast milk might be important for the regulation of bacterial establishment as well as for the donation of nitrogen to the GI tract. The need of nitrogen is known to be higher in newborn infants, as they use it for synthesis of proteins, enzymes etc(11). Bacteria use nitrogen in the large intestine for *de novo* synthesis of amino acids (12), which might be absorbed by the host, but this ability is lost progressively with age. Since the nitrate/nitrite levels in breast milk vary with age, influencing probably the NO generation as well as the nitrogen balance in newborn infant, this should be taken into account when the infant formulas with constant nitrate/nitrite are introduced.

A fundamental remaining issue relates to the role of the bacterial NO generated in the gut. NO is an extremely potent messenger that regulates vital physiological processes in the pico-/nano molar range (13). Thus, even minute production of this gas by gut bacteria could be of biological significance. Judging from the known biological properties of NO, it is not unreasonable that some of the positive effects attributed to probiotics can be explained by formation of NO by these bacteria as has been suggested. NO and other nitrogen oxides generated by lactobacilli from nitrite could help to prevent the establishment of pathogenic bacteria in the gut. Indeed, these reactive nitrogen oxides are highly toxic to many bacteria, including GI pathogens such as *Salmonella, Candida, Shigella, Yersinia* and *E. coli* (14).

We conclude that bacteria residing in the GI tract can be a significant alternative source of NO in the gut in addition to the NO produced in the mucosa. NO generation by gut bacteria differ profoundly from classical mammalian synthesis via NO synthases as bacteria use nitrate and nitrite as substrates instead of L-arginine. Intestinal NO generation can be stimulated by dietary supplementation with substrate and lactobacilli. The NO generation by certain probiotic bacteria can be counteracted with rapid NO consumption by other strains. Future studies will clarify the biological role of the bacteria-derived intestinal NO in health and disease and if an imbalance in generation vs consumption has any significance in the patho-physiology of intestinal disorders. Newly developed unique, minimally-invasive direct measurements of intestinal gases may also be useful to study the dynamics of the microbial colonization process and host-microbial interactions early in life.

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Lecture 2.1.4

Experimental substantiation of new approaches to intestinal microbial ecology correction by means of probiotics

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Introduction: Normal symbiotic microflora is considered as an integral part of the host. In the adult humans the normal microflora is estimated to exceed the total number of mammalian cells by at least an order of magnitude. More than 99% of the commensal gastrointestinal bacteria are obligate anaerobes and belong to 500–1000 species (1). The microflora of the host markedly contributes to the anatomy, physiology and metabolism of man and animal (morphokinetic action, regulation of bacterial and eukaryotic cell-to cell signalling, replication and phenotypic expression of procaryotic and eukariotic genes, apoptosis, angiogenesis, behaviour reactions, biorhythms, participation in metabolism of proteins, carbohydrates, lipids, nucleic acids and other substances, recirculation of bile acids, steroid hormones and other macromolecules, oxidative/antioxidative reactions, etiology and pathogenesis of many infectious and somatic diseases, processing of foods, production of biologically and pharmacologically active compounds, provision of colonization resistance, immune functions, detoxication of exogenic and endogenic compounds and metabolites and so on) (2, 3). Various physical, chemical, biological agents, psycho-social and other stress factors can produce dysbalance of host microflora resulting in different disorders in above mention physiological functions, biochemical and behaviour reactions.
For the last 30–40 years several generations of probiotics containing living gomo- and geteroprobiotic microorganisms (bifidobacteria, lactobacilli, enterococci and others) have been worked out and introduced in practical medicine for correction of microecological disorders in humans and animals. Now-days vast majority of probiotics introduced in medical practice are used for prophylaxis and treatment of infectious diseases caused by pathogenic and opportunistic bacteria and viruses through increasing host colonization resistance, improving immunity, suppressing of microorganism growth and development. As usual probiotic strains used for manufacture of probiotic food additives or probiotic functional foods have been selected on the base of their strong antagonistic activities against disease causative microorganisms. The ability of such probiotic strains interferes into multiple other host physiological and metabolic functions and reactions and possibility of their interactions with other gut symbiotic microbe inhabitants as role have not taken into consideration. Unfortunately the data have appeared that positive effect of probiotics is usually temporary or may be completely absent even in long-term applications. There is information that even lactic acid bacteria can produce host microflora dysbalance or can produce sometimes opportunistic infections (3,4).
Aim: One of the main reasons of probiotic ineffectiveness and appearance of aside effects may be the incompatibility of probiotic bacterial strains with the host resident microbiota. On lactobacillus model the authors of this report experimentally substantiate the necessity attached to selection of probiotics to take into consideration the species, individual and anatomical biocompatibility of probiotic lactobacillus strains with the indigenous lactoflora of future host (transplantation principle).
Results: Joint pair cultivation (*in vitro* on the solid a bit modified MRS medium in the microaerophilic conditions) of 11 industrial probiotic lactobacillus strains and 1079 fresh isolated indigenous lactobacillus strains selected from digestive and vaginal tracts of humans, white rats and mice has shown that all strains investigated might be divided into three groups: compatible, incompatible and synergenic. Antagonistic relationships were predominant among probiotic and fresh isolated lactobacillus strains. In these conditions probiotic strains suppressed the growth and development of more than 60% indigenous lactobacillus strains of human origin. Among probiotic cultures *L.acidophilus* NK-1 and K_3 III 24 (strains used in probiotic food additive "AciLac", Russia) possessed the most spectrum of antagonistic activities and overpowered all test lactobacillus strains investigated. Among indigenous lactobacillus strains this activity depended on species, individual peculiarities of strains, host species and anatomical place of bacterial strain isolation (4). The biocompatibility of resident lactobacillus strains isolated from feces of rodent females and their progeny took place in more than 80% pair strain combinations investigated. Lactobacillus strains isolated from feces of month old animals belonging to the same brood were biocompatible in about 75% combination investigated. Intragastric application of incompatible probiotic lactobacilli (one-day, $1.5x10^{10}$ cfu) to intact adult conventional white mice was accompanied (10 hours later) with short time decreasing of host resident lactobacilli number. Application of incompatible lactobacilli to mice with previously antibiotic eliminated lactoflora was accompanied by temporary colonization of mouse intestinal tract with the new lactobacillus strain/ Additional application of the same animals with compatible homo lactobacillus strain or especially autostrain resulted in more fast displacing of incompatible bacteria previously colonized colon.
In the process of growing in vitro lactobacilli produced various metabolites with molecular mass less and more 12000 D (organic acids, H_20_2, lactocins, lectins, vitamins, peptides, aminoacids and other including non identified substances). In dependence on concentration these metabolites could be as bacterium growth inhibitors or as stimulators of producer and/or other lactobacillus strains. Some metabolites were more active in conditions of neutralization of acids (solid MRS medium with 1% chalk) (5).
Conclusion: To receive fast stable positive probiotic effect and to prevent undesirable aside consequences it is necessary to individualize selection of probiotic strains for each recipient using the widen spectrum of specific *in vitro* laboratory tests.