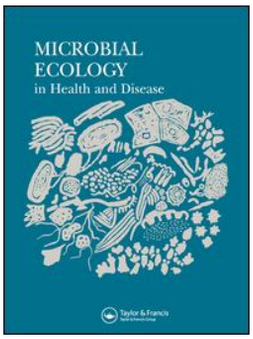

Microbial Ecology in Health and Disease

ISSN: (Print) 1651-2235 (Online) Journal homepage: informahealthcare.com/journals/zmeh20

# Abstracts from the XIII International Symposium on Gnotobiology, held in Stockholm, Sweden, June 19-24, 1999

**To cite this article:** (1999) Abstracts from the XIII International Symposium on Gnotobiology, held in Stockholm, Sweden, June 19-24, 1999, Microbial Ecology in Health and Disease, 11:2, 100-128, DOI: 10.1080/089106099435844

**To link to this article:** https://doi.org/10.1080/089106099435844



Published online: 11 Jul 2009.

# Abstracts from the XIII International Symposium on Gnotobiology, held in Stockholm, Sweden, June 19–24, 1999

Microbial Ecology in Health and Disease 1999; 11: 100–127

## INTRODUCTION

Included in this issue you will find several abstracts covering the up-coming XIIIth International Symposium on Gnotobiology. Why this interest in that part of mammalian ecology? In addition to being personally involved in The International Association of Gnotobiology, part of the answer can be found in my Editorial remarks in Microbial Ecology in Health and Disease 1998;10:1–2:

"Studies in mammals, birds, fish, reptiles and insects with no microbial flora (germ-free individuals) have established basic values for anatomical structures and physiological, biochemical and immunological variables in the macroorganisms themselves. Once such baselines have been established, the normal functions of the flora can be worked out by studies in conventional counterparts. Similar studies in gnotobiotic counterparts, i.e. macroorganisms with a specifically known flora, have shown which species are actually involved. At present, the microbial part of several functions has been clarified, but much more can be done. And the Journal will be there!".

And now, many of the gnotobiologists in the world will convene in Stockholm—and the Journal will be there! It goes without saying that we welcome the lectures to be given in Stockholm as full papers in our Journal.

The key question is: where does gnotobiology go from here? The diversity of the titles within the various sections at the present symposium reflects the power and energy in on-going gnotobiotic research. At the turn of the millennium it seems reasonably safe to forecast that in the future you will find gnotobiotic research involved in many different fields of biology.

*Gnotobiotic research will be in the center of modern ecology*! It is quite clear that, in the future, ecological studies will be furnished with a molecular dimension. Prokaryote–prokaryote and prokaryote–eukaryote crosstalks will be more and more extensively studied and we will learn how cells and microbes whisper and scream on a molecular basis.

*Gnotobiotic research will be in the mid-stream of environmental toxicology*! Even that forecast is more or less self-evident. The list of dietary compounds, drugs and other xenobiotics which are transformed by the host's microflora into more toxic compounds is steadily growing as is the number of compounds studied under gnotobiotic conditions. However, far more compounds will have to be studied more in depth. As an example, think of the long list of 4-fluoroquinolones that are already on the market. These potent antimicrobial drugs may act excellently in the body, however, their fate in the environment is virtually unknown. It is also remarkable that some drugs, such as for instance clofibrate, can be found in high concentrations in sediments in the North Sea. The way from the intestinal tract of patients to the bottom of the sea is completely unknown, and so is their influence on environmental ecosystems.

*Gnotobiotic research will be of increasing importance in modern molecular immunology!* That statement refers to experimental as well as to clinical applications. In parallel with an increasing number of atopic and asthmatic children as well as an increasing number of patients receiving immunomodulating therapy we need a far better understanding of what we are doing and what we are aiming at.

Gnotobiotic research will be in the center of future investigations of genetically modified microorganisms (GEMs)! This statement reflects the mere fact that, in our gnotobiotic system, we do have a proper control of most, if not all, environmental factors. It seems reasonable to assume that in the future, GEMs will not be allowed to be released into Mother Nature until they have been properly tested under gnotobiotic conditions.

*Gnotobiotic research will be of increasing importance in several fields of modern, clinical medicine!* As is evident from the present programme, gnotobiology has already established itself in several fields of clinical medicine, from inflammatory and gene transfer diseases to cancer and transplantation. At the very early beginning of the second century of gnotobiotic research I will allow myself, with the help of a famous statement, to underline that we are just at the end of the beginning of such research. Gnotobiotic animals and technology are here to stay for the next millenium—and our journal, Microbial Disease in Health and Disease, wants to join the gnotobiologists.

*Tore Midtvedt*
Editor-in-Chief

## 2:1 The history and development of gnotobiotic technologies

PHILIP B. CARTER
*College of Veterinary Medicine, North Carolina State University, Raleigh, USA*

The historical development of the technologies for obtaining and maintaining gnotobiotic animals over the past century will be presented using slides and video. Beginning with the early isolation attempts of Nuttal and Thierfelder in Berlin to the development of the Trexler flexible film isolators, with their modern enhancements, gnotobiotic technology will be reviewed. New technological developments, of interest to the experienced gnotobiologist, and film of early human isolation techniques, not seen in a generation, will both be of interest to newcomers to the field. Discussion will focus on the advantages and disadvantages of new developments in isolation and sterilization.

## 2:2 Large germfree animals

JAMES B. HENEGHAN
*Department of Surgery, Louisiana State University Medical Center, New Orleans, USA*

The Germfree Laboratory, LSUMCNO, developed the technology required to rear germfree dogs. The birth of second generation beagles verified the quality of the diet sterilization and isolator maintenance technology. The equipment and labor costs of our procedures will be compared to those developed in the Department of Surgery, University of Wisconsin. When we analyzed our contamination rates they were: highest in dogs, intermediate in rats and lowest in mice. Most contaminations were due to glove failures. Other large germfree animals such as sheep, goats, cows and horses have been reared mainly under the direction of the developer of the flexible film isolator, Phillip C. Trexler. In the next millenium, large germfree animals will be utilized as follows: a) in short term experiments of neonatal microbiology, gut physiology and immunology (Yoon Kim's germfree piglets); b) immuno-suppressed sheep for studies of xenografted human tumors in an animal model whose tumor mass to body weight ratio more closely matches that of patients than that of nude mice or nude rats; and c) laboratories with limited budgets can rear large germfree animals successfully with the older standardized technology (be careful of the gloves and monitor your sterilization temperatures and times). Thus, the future of large germfree animals in the next millenium looks very promising indeed.

## 2:3 The role of the intestinal microbiota on the pathogenicity of *Giardia duodenalis*

MARCIA F. TORRES[1], ANA PAULA UETANABARO, ALESSANDRA COSTA, CELSO ALVES, LUIS FARIAS[2], EDUARDO A. BAMBIRRA[3], FRANCISCO J. PENNA[1], ENIO C.VIEIRA[4] and JACQUES R. NICOLI[2]
*Departments of Pediatrics[1], Microbiology[2], Pathology[3], Biochemistry and Immunology[4], Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*

The exact mechanism underlying the variable clinical symptomatology of the giardiasis which ranges from asymptomatic or transient intestinal complaints to severe long-standing diarrhea and malabsorption is not fully understood but recent results showed that intestinal microbiota is indispensable for the pathogenic expression of the *Giardia duodenalis*. The microbial components responsible for this phenomenon are not known.

Twenty eight facultative and three strict anaerobic microorganisms were isolated from the dominant duodenal microbiota of five patients with symptomatic giardiasis. Groups of germfree mice (GN) were associated with bacterial combinations from each patient. Five days later, all groups were inoculated intragastrically with $10^5$ trophozoites of *G. duodenalis*. Groups of germ-free (GF) and *G. muris*-free (CV) mice were also infected and used as controls. All GN animals were sacrificed 10 days after infection, whereas groups of GF and CV mice were sacrificed at 10, 20 and 30 days. The pathological alterations were higher in CV mice when compared with GF animals but they were intermediary in the GN animals. Total and *G. duodenalis*-specific IgA levels in the small intestine fluid and *G. duodenalis*-specific IgM and IgG levels in the serum increased during the infection and were higher in CV mice for all the times when compared with GF mice.

The results showed that the intestinal microbiota plays a role on the pathogenicity of *G. duodenalis* and some combinations of intestinal microbial components can develop partially this function. However, none of these combinations was able to stimulate the protozoan pathogenicity in a similar way as for the whole intestinal microbiota.

Supported by CNPq, FAPEMIG, FINEP and PRPq.

## 2:4 Gnotobiotic lambs: a powerful tool for studying the effect on microbial activities of *Saccharomyces cerevisiae* I-1077, a probiotic yeast used in ruminant nutrition

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The objective of our study was to investigate the role of *Saccharomyces cerevisiae* CNCM I-1077, a probiotic for ruminants, on establishment of cellulolytic bacteria, plant cell wall degradation and fermentations in the rumen of lambs. This study was carried out with gnotobiotically-reared lambs harbouring a defined cellulolytic microbial community composed of bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* as major fibre-degrading species). Naturally-born lambs were placed in steril isolators 24 hours after birth. At this age, their rumen is already colonized by a diversified bacterial anaerobic microflora but they are free of cellulolytic microorganisms (bacteria, fungi, protozoa). The days after birth pure cultures of the three cellulolytic species named above were inoculated to the animals.

Cellulolytic bacteria became established more rapidly at a higher level in presence of SC. Their population remained stable in the rumen of lambs receiving SC compared to control animals, when the physico-chemical conditions of the biotype was altered (canulation). Several polysaccharide and glycoside hydrolases of solid digesta-associated bacteria exhibited a greater specific activity in presence of SC, and degradation of weat straw measured by the nylon bags method was significantly increased (15%). A lower ammonia concentration in the rumen of lambs receiving SC was also observed, which suggest the presence of more active microorganisms in these animals, since ammonia is the preferential source of nitrogen for most of the rumen microbial species. These data demonstrate that SC I-1077 is a good tool to stimulate the development of the rumen callulolytic microflora and the activity of the microbial community in the rumen of young ruminants.

## 2:5 Rearing of axenic and gnotobiotic pigs

P. WALLGREN
*National Veterinary Institute, Uppsala, Sweden*

Axenic (sterile) pigs are delivered by caesarean section, preferably made as late as possible in the gestation. The piglet-incubators must be sterile and ought to be kept around 33°C at delivery, a temperature that is decreased by 1°C every second day until 25°C is reached. The piglets may repeatedly be offered small amounts of sterilised bovine milk (7% fat). The consumption ought be approximately 70 ml on the day after delivery, and should be increased by 20–30 ml per day during the first 2 weeks of life. From that time the feed can gradually be exchanged to sterilised dry feed. It should be remembered that the growth of the animals is retarded due to spoiled proteins and vitamins of the feed following sterilisation. Axenic piglets become gnotobiotic if they are exposed to a defined microflora (and nothing else), often given per os on living day 2 or 3. Both categories of animals require rearing in incubators The number of transportations to and from these incubators should be minimised in order to reduce the risk for contamination. For long-term experiments, switching of pigs to new incubators rather than cleaning facilities in use is recommended. Specific demands should be fulfilled before making experiments in axenic/gnotobiotic pigs. Not only due to effects of the feed, but also since these animals do not have been exposed to ubiquitous microorganisms nor have received any passive immunity via colostrum.

## 2:6 Treatment of children in conditions of gnotobiological isolation. 10-years experience

V. KOLYADENKO, B. SHENDEROV, YU. TSAREVA
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Under the conditions of gnotobiological isolation with laminar flow of sterile air (Standart 209, USA) bolstered automatically 4393 patients at the age from 3 months to 14 years with a various pathology (bronchial asthma, obstructive bronchitis, pneumonia, mucoviscidosis, Lyell's disease, atopic dermatitis, sepsis, nonspecific ulcerative colitis, whooping-cough) were treated. Next indexes and parameters were investigated: duration of treatment; time of temperature normalization, BOS liquidation; acid–base balance; condition of humoral and cell immunity, thyroid gland; lipid peroxidation and antioxidative active activity of blood; morphofunctional and microbiological condition of respiratory and gastrointestinal tract mucosa, and others. The results testified about high clinical efficiency of the children treatment in the conditions of gnotobiological isolation. The reduction of treatment terms was marked and they were 2–4 times less in comparison with traditional treatment. Vast majority of parameters and indexes investigated restored to normal at 78% patients. The mechanisms of clinical efficiency of treatment in conditions of gnotobiological isolation will be discussed.

## 3:1 Progress in laboratory animal science

C. MAX LANG
*Department of Comparative Medicine, M. S. Hershey Medical Center, Pennsylvania State University, Hershey, PA, USA*

Significant progress has been made in laboratory animal science during the last several decades, especially in husbandry and environmental factors that can affect the interpretation of research data. However, the relatively new, and intensified, focus on transgenic animals requires a re-examination of that progress; implementation of cost-effective means of housing such animals; development of new technology; expansion of gnotobiology research; and training in animal pathobiology.

## 3:2 **The Philip C. Trexler lecture.** The role of the flora in cytokine responses

EDWARD A. HAVELL, PRESIDENT, ASSOCIATION FOR GNOTOBIOTICS
*College of Veterinary Medicine, North Carolina State University, Raleigh, USA*

My research interest has centred on the roles of the interferons (IFN) and other cytokines (e.g. tumor necrosis factor [TNF]) in host resistance to infectious diseases. Interest in IFN originated from the results of experiments using gnotobiotic mice which showed that the composition of the intestinal microflora influenced the ability of mice to produce IFN. Subsequent research on IFN led to the discovery of IFN-β which was show to be antigenically distinct from the two other IFN classes (α, γ). It was also observed that mice injected with IFN or TNF showed enhanced resistance to bacterial infections. Moreover, it was also found that mice produced IFN (α, β, γ) and TNF early during a number of bacterial infections. To analyze the possible roles of IFN and TNF in innate resistance and adaptive immunity in mouse models of infectious diseases, specific-IFN-γ antibody and anti-TNF antibody were produced and injected into mice undergoing infections with different pathogens. It was reasoned that if a cytokine functioned in host resistance to a pathogen, then by blocking the cytokine's action *in vivo* by its specific antibody would result in enhanced pathogen numbers in the infected tissues of the host. Indeed, it was found that anti-TNF antibody or anti-IFN-γ antibody treatment exacerbated a number of bacterial and protozoan infections in mice. For example, in *Listeria monocytogenes* infections in mice, TNF was shown to be important early in innate resistance, whereas IFN-γ was determined to be important later in infection.

## 3:3 Germfree animals and techniques in surgical research: An update

J.B. HENEGHAN, F.C. NANCE, S.G. LONGORIA and I. COHN, JR.
*Department of Surgery, Louisiana State University Medical Center, New Orleans (LSUMCNO), Louisiana, USA*

Since the surgical research in the Germfree Laboratory, LSUMCNO has been reviewed, we will summarize only the most significant studies and will concentrate on current research and the status of gnotobiotic animals and techniques for surgical research in the next millenium. The value of germfree research for surgical problems has centered around the study of the role of the microbial flora in intestinal crises such as strangulation obstruction, bile peritonitis, hepatic artery ligation, pancreatitis and hemorrhagic shock. Gnotobiotic isolation technology has been very successful in preventing infectious disease in immuno-compromised patients and experimental animals. Currently our work used gnotobiotic containment technology to protect nude rats and mice with xenografted human tumors from infection. The same technology protected individual experimental animals from environmental toxins during low level exposure in quantitative feeding studies of lead bioavalablity. In the new millenium, the following surgical problems will benefit the most with the application of gnotobiotic technology: isolation of immuno-compromised patients and experimental animals and short term studies of neonatal immunology and intestinal pathology.

## 3:4 Influence of psychological stress on the fecal carriage of indicator bacteria

E. Bezirtzoglou[1], M. Konstandi[2], C. Voidarou[1], D. Kostakis[2] and M. Marselos[2]
*Department of Microbiology*[1] *and Department of Pharmacology*[2], *Medical School*, *University of Ioannina*, *Ioannina*, *Greece*

It is well accepted that an organism under the influence of stress is less capable of defending iltself against infectious, cancer cells and other disease factors. The detoxifying mechanisms of the body are affected as well, a fact, which may lead to severe health problems. E. coli and C. perfringens are recognized to be the typical fecal indicator bacteria, which may cause serious disease states when the bacterial balance is disturbed. Our aim was to assess whether repeated mild unpredictable stress used as a model of psychological stress, modified the carriage of the fecal indicator bacteria. Double fecal samples were collected from adult male Wistar rats (Kuo/Ioa/rr). Rats were exposed to repeated mild unpredictable stress as follows: 1st day: cold swimming at +4°C for 2min; 2nd day: food deprivation for 24hrs; 3rd day: exposure to white noise for 1hr; 4th day: restraint stress for 2hrs; 5th day: electric footshock of 1.5mA for 20min (1 shock per 30sec); 6th day: restraint stress for 2hrs and finally the 7th day: placement for 5min in the cage where previous electric footshock took place (no footshock, however, was performed). The stress task took place between 09:00 and 12:00 in an isolated environment. Feces sampling took place in the beginning of the experiment before the first stress task and the 2nd, 3rd, 5th and 7th day immediately after stress. In addition, the effect of psychological stress in the fecal flora was evaluated the 1st, 3rd and 10th day after the termination of stress. All analyses were compared to controls. Serial dilutions were performed in Ringer followed by spreading in Mac Conkey agar incubated overnight at 37°C. An aliquot of the solutions was heated for 10min at 75°C, followed by spreading in Lactose-Sulfite (LS) broth. Numbers of C. perfringens were estimated by performing decimal dilutions in the LS broth incubated overnight in a waterbath at 46°C. Characteristic colonies of E. coli were counted and identified by using Api 20E system. Numbers of C. perfringens were estimated and compared with those of E. coli. Concerning the anaerobic bacterial indicator C. perfringens, vegetative forms were found under after stress in higher numbers compared to controls. Sporulated forms of C. perfringens were also isolated in high numbers. Under stress, the population of E. coli presented fluctuations but remained always in low numbers. Taken together, our results suggest that repeated mild unpredictable stress may serve as a critical regulator for the growth of C. perfringens, implicated in serious septic complications.

## 3:6 Gnotobiotics with microisolators utilizing ventilated cages and automatic watering

Robert S. Sedlacek
*Experimental Radiation Oncology*, *Massachusetts General Hospital*, *Boston*, *MA*, *USA*

Static microisolators have proven effective in preventing cage to cage and room to cage spread of microorganisms.

We designed experiments to test the efficacy of ventilated microisolators using *Staphylococcus epidermidis* when cages were placed opposite, adjacent or when associated cages were switched rack positions with gnotobiotic DF control cages.

Automatic water experiments were performed replacing conventional mice with DF mice over a period of 10 months while maintaining full use of the rack. We developed a recirculated water system employing clean water, UV and HCl. Six cages of mice infected with Sendai, MHV and MVM were placed at the end of the water manifold and later transferred to the beginning.

In the air experiment all DF cages remained free of *Staphylococcus epidermidis*. In the water tests, no additional bacterial association took place in the DF mice until week 28 when four cages became infected with a Bacillus. Tests confirmed that the batch of irradiated diet was unsterile. The four positive cages and those coming from them were removed. The change over to DF continued until week 43 when all 126 cages were confirmed DF.

Serology confirmed Sendai and MHV infected but MVM failed to infect. The infected cages were placed at the end of the water manifold for six weeks. Samples were taken and the infected cages moved to the start of the water manifold. Six weeks later serology confirmed the DF mice were free of Sendai and MHV.

## 4:1 Prokaryote–prokaryote transfer of genetic material in the GI-tract of gnotobiotic animals

A Sundsfjord, KH Dahl, EW Lundblad, TP Røkenes, GS Simonsen, B Haldorsen, MR Mikalsen, P Johnsen, T Midtvedt, Ø Olsvik
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Much of the bacterial world is represented at some level in the microflora of the mammalian intestinal tract; resident flora, transient colonizers from water, soil and food as well as pathogens. The conditions in the digestive tract favour gene transfer between these bacteria. Antibiotic resistance determinants have been used as a system to study gene flux between bacteria, because they are easily selected and traced. Although both transduction and transformation may be involved in such gene transfer, conjugation combined with transposition seems to be the most likely transfer mechanism due to its broad host range. The power of a germ-free animal system in the study of horizontal gene transfer in prokaryotes lies in the ability to control the environmental conditions.

Gnotobiotic animals have been used as an experimental system to demonstrate transfer of antibiotic resistance genes between related as well as distantly related bacteria; i.e. trans-Gram conjugal transfer by broad host range plasmids or conjugative transposons. It seems as if almost any bacterium has the potential to transfer genes to any other bacterium on the assumption that a functional gene-transfer element is present in the donor strain. The fate of DNA in the mammalian GI tract as well as the ability of intestinal microflora to pick up, stabilize and express free DNA has not been adequately examined in vivo. This is basic information in the risk assessment of genetically engineered organisms with antibiotic resistance gene markers, which can be obtained in germ-free animals systems.

VanA-type vancomycin resistant enterococci (VRE) are emerging human pathogens colonizing the intestinal tract of human hospital patients worldwide as well as outpatients, farmers and farm animals in several European countries exposed to the use of avoparcin (glycopeptide antibiotic) as a growth promoting feed additive in animals. Our laboratories have examined the ability of VRE from different reservoirs to transfer their resistance genes in vitro and in germ free animals under different experimental conditions in order to understand transfer mechanisms as a potential target for intervention and as a prerequisite for long-term persistence. Experimental design and preliminary results will be presented and discussed.

## 4:2 In vivo transfer of the *satA* gene encoding streptogramin a resistance between isogenic strains of *Enterococcus faecium* in the mammalian gastrointestinal tract

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[2]*Danish Veterinary Laboratory, Copenhagen V Denmark*

We have previously shown that transfer of the *satA* gene between isogenic strains of *Enterococcus faecium* may take place in the gastrointestinal tract using a group of five gnotobiotic rats. Due to the important aspects of these findings the study was repeated with an additional group of five gnotobiotic rats. Five germ-free rats dosed p.o with a single high dose of $7 \times 10^8$ cfu of a streptomycin resistant recipient *E. faecium* BM4105-Str. One week later the same rats were dosed with $2 \times 10^8$ cfu of the rifampicin, fusidin and virginiamycin resistant donor *E. faecium* AHA15(satA). Shortly after, using the specific antibiotic resistance pattern for isolation on agar plates transconjugant *E. faecium* BM4105-Str(satA) were identified in high numbers in faecal samples. High concentrations of the transconjugants were observed the next 18 days The presence of the *satA* gene was verified by PCR analysis. The study confirms the previous observation that transfer of the *satA* gene may take place under experimental conditions in the mammalian gastrointestinal tract. This indicates that a similar transfer may take place under natural conditions.

## 4:4 **Rune Grubb lecture** Rheumatoid arthritis-a gene transfer disease?

Tore Midtvedt
*Medical Microbial Ecology Karolinska Institute Stockholm Sweden*

In 1956, the Swedish scientists Grubb and Laurell convincingly showed that production of immunoglobulins of the Gm type was under Mendelian control and not determined by templates of antigens. Over the years, not a single authenicated example of a child possessing an allotype which both parents lacked has been encountered among many thousands of pairs.

However, the astonishing finding that in patients with rheumatoid arthritis (RA), an immune response to other individual Mendelian allotypes are prevalent, although RA is generally considered an autoimmune disease, prompted the late Rune Grubb and his co-workers to put forward the hypothesis that RA is not initially an autoimmune disease but a gene transfer disease (1). They are bringing up good evidence for putting the herpesvirus family into focus as vectors. In a challenging conclusion they write that ''we consider that the concept of gene transfer disease has wider applicability than RA. Scleroderma may be classed as a gene transer disease, accepting Nelson's interpretation. Gene transfer may obviously involve other viruses than the herpes viruses and other polymorphic traits than immunoglobulins. Some possible cadidates are type I diabetes and multiple sclerosis''.

Gnotobiology should be the place to study prokaryote transfer of eukaryote genes. Rune Grubb made the challenge-let us go for the solutions.

1. Grubb R, Grubb A, Kjellen L, Lycke E, Åman P. Rheumatoid arthritis-a gene transfer disease. Exp Clin Immunogenet 1999;16:1–7.

## 5:1 Studying host-microbial cross-talk—to hear when nature screams and whispers

Per Falk
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Interactions with prokaryotes are essential for our existence, but may also be detrimental to our health. The gastrointestinal tract exhibits the greatest complexity of all mammalian ecosystems, and most of the diseases linked to indigenous bacteria likely emerge in the gut. These include infections, inflammatory conditions, allergies, and cancer. Studies of host–microbial interactions have focused on pathogenesis. However, parasitic encounters with bacteria are rare compared with the perpetual cross-talk with indigenous bacteria. Studies of mammalian ecology is a challenge as it lacks the dramatic phenotypes explored in pathogenesis. The ecosystem constitutes an immense number of signaling events, involving the epithelium, the indigenous microflora, the gut-associated immune system, and other submucosal cells, e.g. the enteric nervous system. Function or dysfunction, i.e. health or disease, is the result of these interactions combined.

Characterization of host-microbial interactions under non-pathogenic conditions requires the assessment of cellular functions in the absence of microorganisms. This provides the 'baseline' for subsequent evaluation of how exogenous factors influence on establishment and maintenance of gastrointestinal functions. The germ-free state represents the only tool for controlling the environment that a mammal is exposed to. Our understanding of gastrointestinal ecology will expand when physiology and biochemistry are combined with genetics and germ-free technology. This will have profound impact on our way of looking at a number of hitherto poorly defined diseases, and pave the way for novel strategies for treatment and prevention of infectious diseases and immunopathology.

## 5:2 A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem

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Little is known about how members of the normal microflora interact with their mammalian hosts to establish mutually beneficial relationships. Gnotobiology represents a key technology for understanding the molecular basis of host–microbial cross-talk. We have developed a gnotobiotic model to study one example of such cross-talk. *Bacteroides thetaiotaomicron* is an abundant and genetically-maniputatable normal inhabitant of the mouse and human intestine. We have shown previously that this anaerobe orchestrates a program of fucosylated glycan production in the ileal epithelium when it is introduced into germfree mice. A mutant *B. thetaiotaomicron* strain that cannot utilize fucose is unable to elicit this program, suggesting a link between fucose utilization and the ability to signal host production of hydrolyzable fucosylated glycans. To understand this connection, we have constructed a series of isogenic strains, each containing a disruption of one of the fucose utilization pathway genes. Analysis of ex-germ-free mice colonized with these strains reveals that a bacterial repressor (FucR) coordinates expression of the *B. thetaiotaomicron* fucose utilization operon with expression of another locus that regulates fucosylated glycan production in intestinal enterocytes. This coordination is accomplished by fucose acting through FucR as an inducer at one locus and as a corepressor at the other locus. Thus, FucR functions in a novel way as a molecular sensor of fucose availability. *B. thetaiotaomicron*'s evolution of a system that tightly couples its immediate nutritional requirements to production of a host-derived energy source is consistent with its need to create a niche within the highly competitive intestinal ecosystem.

## 5:3 Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice

TOR SAVIDGE
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To investigate the roles of astroglial cells, we targeted their ablation genetically. Transgenic mice were generated expressing herpex simplex virus thymidine kinase from the mouse glial fibrillary acidic protein (GFAP) promoter. In adult transgenic mice, 2 weeks of subcutaneous treatment with the antiviral agent ganciclovir preferentially ablated transgene-expressing, GFAP-positive glia from the jejunum and ileum, causing a fulminating and fatal jejuno-ileitis. This pathology was independent of bacterial overgrowth and was characterised by increased myeloperoxidase activity, moderate degeneration of myenteric neurons, and intraluminal hemorrhage. These findings demonstrate that enteric glia play an essential role in maintaining the integrity of the bowel and suggest that their loss or dysfunction may contribute to the cellular mechanisms of inflammatory bowel disease.

## 5:4 Comparative study of the ability of a genetically modified *Lactococcus lactis* strain to survive and colonize the gut of rats after oral administration

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Genetic modification of a micro-organism may influence the ability to survive passage through the gastrointestinal tract and could potentially affect the health of humans. To obtain data for a risk assessment of two genetically modified *Lactococcus lactis* strains, their ability to survive and colonize the intestine in the rat and the effect upon the intestinal flora, was investigated. All investigations were performed relatively to the parental strain. The different strains were given in a single high dose to separate groups of rats for a period of seven consecutive days followed by an observation period of 21 days. None of the modified strains or the parental reference strain were found to colonize the intestinal tract. However, the modified strains differed from the parental strain in having a higher survival rate. Changes in the composition of the microflora and changes in the concentrations of volatile fatty acids in faecal samples were also observed. The rats given the modified strains did not show any macroscopic changes compared to the parental type at interim and final sacrifice, nor did clinical observations indicate any adverse effects.

## 5:5 A soluble factor from *Bacteroides thetaiotaomicron* VPI-5482 specifically increases the galactosylation pattern of HT29-MTX cells

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Intestinal bacteria have been shown to modulate the production of particular glycoconjugates in the mice intestine. The aim of this work was to set up an *in vitro* model to study the capacity of *Bacteroides thetaiotaomicron* strain VPI-5482 to change a specific glycosylation process in cultured intestinal cells (HT29-MTX) via a mechanism, which involves a soluble factor. The possibility of transposing the *in vivo* results to an *in vitro* model seems essential to further study the molecular mechanisms of these interactions.

Early and late differentiated HT29-MTX cells were grown in the presence of 10 and 20% spent culture supernatant from the *B. thetaiotaomicron*, during 10 days. Glycosylation processes were followed using a large panel of lectins and analyzed using confocal microscopy and Western blotting techniques.

A *B. thetaiotaomicron* soluble factor modified specifically the galactosylation pattern of HT29-MTX cells, whereas other glycosylation steps remained mainly unaffected, visualized using both RCA and Jacalin galactose specific lectins. The increased expression of galactose only slightly depends on the differentiation stage of cells and concentration of the bacterial medium used.

Our *in vitro* model allowed to study the cross-talk between single bacteria and intestinal cells. The results obtained demonstrated that *B. thetaiotaomicron* produces a still uncharacterized soluble factor capable of modifying a specific glycosylation process in intestinal cells. The galactosylation process appears to be a target of this communication, thus uncovering a new window to study the functional consequences of cooperative symbiotic bacterial-host interactions.

## 5:6 Intestinal cell kinetic studies in ex-germfree rats monocontaminated clostridium difficile

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The aim of the study was to examined whether-and what extent-a short term establishment of *Clostridium difficile* in young, male germfree (GF) rats influenced upon intestinal cell kinetics. The bacterial strain was established in a cohort of 35- days old GF rats. After 3 or 7 days of establishment, the animals received vincristine 1 mg/kg to arrest and preparations for microscopic examinations were taken exactly 4 hrs afterwards. The total number of mitotic cells and cell nuclei were counted in the left column of 30 consecutive well-oriented crypts in 4 sections of the small and large intestine, respectively.

After 3 days of monocontamination, no sign of microscopic inflammation was observed neither in the small nor in the large intestine. The mitotic index was increased in all sections except one (ileum) 3 days after monocontamination. After 7 days of monocontamination, signs of mild infection could be seen. The mitotic index reduced to about 1/4 of the original values throughout the whole small intestine. In the large intestine, the values were around half of the original values in all sections except cecum where the mitotic index was significantly increased. The results clearly indicate that *Cl difficile* do interfere profoundly with intestinal cell kinetics in ex-germfree rats.

## 6:1 Gut microflora metabolism—what can the microbes do?

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The colonic microflora has been shown to possess a wide range of metabolic activities, resulting in the formation of substances with carcinogenic, genotoxic, tumour-promoting and anti-carcinogenic activity. For example, the enzyme ß-glucuronidase is involved in the release in the colon from their conjugated form of a number of dietary toxicants and carcinogens, including polycyclic aromatic hydrocarbons. The bacterially-catalyzed reaction of nitrite with secondary amines and amides can lead to the formation of N-nitroso compounds, many of which possess mutagenic and carcinogenic activity. A major role for the intestinal microflora has been identified in the formation of potential tumour-promoters, such as secondary bile acids, ammonia, phenols and

cresols. In general species of *Bifidobacterium* and *Lactobacillus*, in contrast to bacteroides eubacteria and clostridia, have low activities of many of these enzymes suggesting that increasing the proportion of such lactic acid producing bacteria in the gut could modify, beneficially, the levels of xenobiotic metabolising enzymes. There can be marked interindividual variation in gut microflora activities in human subjects. For example, the microbial conversion of the dietary isoflavonoid daidzein to equol occurs in only 35% of subjects, the remainder excreted virtually none. In addition to the direct effects of the microflora on metabolism of xenobiotics, gut bacteria can influence mammalian pathways of metabolism. Studies in germ-free rodents have shown that the presence or absence of a microflora can modify the hepatic activation of the carcinogen aflatoxin B1 and can affect the activity of glutathione transferase in the colonic epithelium

I thank the EC (FAIR CT-95-0894 and CT-94-0933) for financial support.

## 6:2 A new model to study hydrogen metabolism of human intestinal microflora using gnotobiotic rats

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Hydrogen ($H_2$) is produced during fermentation in the intestinal tract of humans and animals. $H_2$ is excreted in breath and flatus, but to a large extent utilised by microorganisms through pathways which may include methanogenesis, acetogenesis, and sulphate reduction. The study of $H_2$ excretion affords an insight into the *in vivo* metabolic processes of $H_2$ formation and $H_2$ utilisation by the intestinal microflora and provides information of both clinical and nutritional relevance.

Here we report on a new animal model by which the total $H_2$ excretion can be monitored routinely in the gnotobiotic rat fed a chemically defined diet. The experimental set-up consists of the isolator containing a chamber for an experimental animal and a life-support system with a $H_2$ remote measuring head outside the isolator connected to it. Prior to the experiment, a test substance was administered to the animals and $H_2$ excretion was measured before and after monoassociation with $H_2$ producing bacteria and after subsequent diassociation with $H_2$ oxidising bacteria (human isolates). After the technical description, the system is validated by investigating the effect of lactulose on rats monoassociated with a $H_2$ producing *Clostridium perfringens* strain. A minimal yield of 156 ml $H_2$/g lactulose can be calculated from these data. In similar experiments using a $H_2$ producing *Escherichia coli* strain the decrease of the $H_2$ excretion upon diassociation with a $H_2$ utilising microorganism is also characterised. Experiments investigating the mechanisms how a particular $H_2$ utilising microorganism prevails in competition with others are in preparation.

## 6:3 Bacterial activity, measured by $PCO_2$, in the caecum of mice and the effects of diet composition

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Measurement of $PCO_2$ in the lumen and on the serosa of caecum was used as a non-specific measure of bacterial metabolic activity in conventional *HsdHan:NMRl*, *Bkl:NMRI* and *Hsd:ICR mlce*, as well as in germfree and conventionalized germfree KI:NMRI mice. $PCO_2$ r was recorded with Severinghaus-type microelectrodes *in vlvo* (during *Hypnorm*$^R$/*Dormicum*$^R$ anaesthesia) and *post mortem*. Different commercia1 diets (*RM1(E)SQC*, SDS, England and R36, Lactamin, Sweden) and custom made rodent diets were used in the *HsdHan:NMRI* mice, to test the effects of diet.

$PCO_2$ levels were up to $55 \pm 4$ kPa (mean $\pm$ SE) in the caecal lumen and $27 \pm 3$ kPa on the serosa side in conventional mice maintained on *RM1(E)SQC* diet. Flushing of the caecum resulted in significantly lower serosal and luminal $PCO_2$ levels of 8–10 kPa. Caecal $PCO_2$ was significantly lower in germfree mice ($9 \pm 1$ kPa) on R36 diet. Caecal $PCO_2$ increased significantly after introduction of normal bacterial flora into germfree mice. After death, serosal $PCO_2$ of the caecum increased rapidly in normal mice. These data indicate that the intestinal bacteria produced the majority of the caecal $PCO_2$.

After 14 days maintenance on a synthetic diet, composed of 73% glucose as the only carbohydrate source, $PCO_2$ was $23 \pm 1$ kPa in the caecal lumen and $12 \pm 1$ kPa on the serosa in *Hsd:Han:NMRI* mice. When R36 diet was used, was $38 \pm 3$ kPa in the caecal lumen and $14 \pm 2$ kPa on the serosa in *Hsd:Han:NMRI* mice.

The in vivo $PCO_2$ levels recorded in conventional mice are sufficiently high to create a state of gas supersaturation. We propose that the thin caecal wall and the sufficient vascular clearance of $PCO_2$ in mice are the cause of the high serosal $PCO_2$ levels in mice

## 6:4 Establishment of hydrogenotrophic acetogenic bacteria in the rumen of gnotobiotically-reared lambs

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In the rumen, methanogenesis is the major mechanism of hydrogen-reutilization during fermentation of the feed. Methane is eructated and represents a loss of carbon and energy for the ruminant and a pollution for the atmosphere. In contrast, in many gut microbial ecosystems, acetogenic bacteria (acetogens) rather than methanogens function as the hydrogenotrophs. Acetogens which can convert $CO_2$ and $H_2$ to acetate are present in the rumen but the size of their population is usually small and their metablic capabilities and their ecological significance are poorly understood. To understand why these species are not naturally competitive with the methanogens for $H_2$-utilization in various types of gnotobiotically-reared (meroxenic and gnotoxenic) lambs harbouring a more as less defined and diversified microflora. We have particularly studied the establishment and development of an acetogenic population in the rumen of these animals in absence of competition with methanogens. The main results were the following: 1) acetogens were able to colonize the rumen of germ-free lambs at a level much more higher that usually observed in conventional lambs; 2) in meroxenic lambs, harbouring a methanogen-free complex microflora, the size of the acetogenic population was approximately the same than that usually found in conventional ruminants. Inoculation of methanogens to these meroxenic lambs did not alter the stability of the acetogenic poulation. Therefore, these results suggest that the size of the rumen acetogenic population is not only determined by competition with archæa methanogens but that acetogens probably interact with other non-methanogenic hydrogenotrophs.

## 6:5 Role of gut *Lactobacillus plantarum* in controlling human urinary oxalate

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Oxalates are widely distributed in diets and potentially toxic for human tissues. Since 1940 the hypothesis that gut microflora plays and important role in the metabolism of oxalate has been known. Some experimental and clinical data have been accumulated to support this thesis. Nowadays two groups of gut microorganisms (Oxalatobacter and Lactobacilli) able to regulate the host oxalic acid pool investigated in detail.

The present report shows that some strains of *Lactobacillus plantarum* of human gut origin can regulate the level of oxalic acid pool in urine of persons with hyperoxaluria. The experimental group included 20 persons receiving O-LACT (functional food product on the base of milk fermented with *Lactobacillus plantarum* possessing oxalate degrading activity) daily for 4 weeks. The control group (10 persons) got the sour milk with *lactobacilli* without oxalate degrading activity. The concentration of oxalic acid ions in urine and gut microflora condition were determined before and after oral probiotic therapy. Comparisons of gut microbial ecology and urinary oxalate excretion determine before and after *lactobacilli* intake in experimental and control groups of stone formers showed that intake of living oxalate degrading *lactobacilli* was accompanied with normalization of host gut microflora, decreasing of urinary oxalate excretion, improving some biochemical serum and urine indexes.

## 6:6 The effect of inulin consumption on the intestinal flora of healthy volunteers measured by fluorescent *in situ* hybridization

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We have developed fluorescent 16S rRNA targeted oligonucleotide probes for numerically important groups of bacteria in the human intestine. These probes have been used for whole cell fluorescent *in situ* hybridization (FISH) to study the 'baseline' composition of the human intestinal flora (Franks et al., Appl. Environm.Microbiol. 64 (1998) 3336).

In the present study, these probes have been used to study the effect of inulin consumption on the intestinal flora of 9 healthy volunteers. Supplementing the diet for 14 days with 9 g inulin (a mixture of oligo- and polysaccharides composed of fructose units with a degree of polymerization between 2 and 60) per day did have individual-related effects on the number of bifidobacteria. Relative effects ranged from 0.6 to 6.1 (mean 2.7) times the original number of bifidobacteria.

In general, volunteers with high initial numbers of intestinal bifidobacteria tend not to respond to inulin treatment, while volunteers with relatively low initial numbers of bifidobacteria tend to respond with an increase in bifidobacteria. This may imply the existence of an ecological niche for the genus Bifidobacterium in the intestinal ecosystem.

## 6:7 Determination of protein degradation products in faecal sarnples from breast-fed and formula-fed infants

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Although differences have been reported in the microbial composition of the flora between breast fed and formula fed babies, little is known of the consequences of these differences in terms of bacterial metabolism in the gut We have investigated effects of diet on the faecal concentration of ammonia, phenol and p-cresol which have been shown to have toxic effects both locally on the intestinal mucosa and systemically.

Mothers of new-born babies in Glasgow were approached within two days of birth and those who were exclusively breast feeding or exclusively formula feeding were invited to participate in the study.

The concentrations of phenol and p-cresol (analysed by gas chromatography) and ammonia (analysed spectrophotometrically) were determined on 91 faecal samples from breast fed babies and formula fed babies aged 1 week to 1 year. Faecal arnmonia concentrations ranged from 0–22 μmol/g which are substantially lower than those in adults (40 μmol/g). The concentrations of ammonia increased gradually with age, especially during weaning. It also appeared that breast fed infants had lower faecal concentrations of ammonia than formula fed infants. However, this effect was less evident once weaning commenced. Similar changes were seen for phenol and p-cresol concentrations.

This work was funded by the EU under contract FAIR CT97-3181 (DG12-SSMI).

## 7:1 The MAC/GAC concept: principles and consequences

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The gastrointestinal flora in mammals can be investigated in several different ways. In the past, most reports had dealt with the composition of the flora, and great efforts have been made to isolate, identify and enumerate the hundreds of species now known to be present. New molecular methods are adding valuable information about the composition. Another approach is to study the metabolic capability of the flora, i.e. "what can the microbe do?"-and that approach was addressed in the previous section. A third approach is more directly to study the functional status of the flora, i.e. "what have the microbes done?". In such studies, two terms-Microflora-Associated Characteristic (MAC) and Germfree Animal Characteristic (GAC)—have been shown to be of considerable value. A MAC is defined as the recording of any anatomical structure or physiological, biochemical or immunological function in a macroorganism that has been influenced by the microflora, When microorganisms that influenced the variable under study are absent, as in germfree animals or newborns or in relation to ingestion of antibiotics, the structures and functions are defined as GACs.

In the lectures to follows, the MAC/CAC concept will be applied on several different fields of research. The bottom line is that MACs and GACs are principally the same in all mammals. There might be quantitative, but never qualitative differences.

## 7:2 The MAC-GAC concept: application on human material

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The MAC-GAC concept have been used for studies of different human materials, i.e., in materials from humans during antimicrobial therapy, where it is possible to see that each amtimicrobial drug have its own MAC-GAC profile.

In material from healthy humans admitted to a normal health control, we found differences with regard to age and sex in some of these parameters and these results will be presented and discussed.

## 7:3 The effect of faecal enema on six microflora-associated characteristics in patients with antibiotic-associated diarrhoea

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Antibiotic-associated diarrhoea (AAD) may range from mild disturbances to severe pseudomembranous colitis. Antibiotics may affect several intestinal Microflora-Associated Characteristics (MACs), however, each drug has its own profile. In the present study we investigated MACs in 32 patients when admitted to the hospital for severe AAD. The different MACs were: formation of coprostanol and urobilinogen, degradation of trypsin and β-aspartylglycine and investigation of the mucin and short-chain fatty acids (SCFAs) profoile with gas chromatography, electrophoresis and spectrophotometry. Nine patients were followed more extensively before and after administration of an enema containing faecal microflora from a healthy person on a 'Western diet'. At admittance to the hospital, the conversion of cholesterol to coprostanol and the concentration of urobilinogen and trypsin were significantly reduced in comparison with healthy persons. The patients showed also significant disturbances in faecal SCFA pattern and the total amounts of the SCFAs were reduced. The pattern of mucin was altered, but the β-aspartylglycine remained the same as in healthy persons. In most of the patients the faecal enema treatment normalised rapidly the different MACs studied. We found that the microflora in the AAD patients showed significant disturbances in most MACs as compared with healthy persons. The administration of a human faecal enema modified these changes and relieved diarrhoea, usually within 4 days.

## 7:4 Microflora associated characteristics in faeces from allergic and non-allergic children

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Background: The prevalence of allergic diseases seems to have increased particularly over the past 30–40 years. It has been suggested that a reduced microbial stimulation during infancy would result in a slower postnatal maturation of the immune system and development of a disturbed balance between Th1 and Th2 like immunity. The gut flora is, quantitatively, the most important source for such stimulation. The aim of the study was to compare the gut microbial flora in allergic and non-allergic infants, through analysing microflora associated biochemical markers.

Materials and methods: Faeces samples from 25 allergic and 47 non-allergic Swedish, 1-year old infants were selected from a prospective study. Microflora associated characteristics (MAC) were assessed by determining the concentrations of 8 different short chain fatty acids and the conversion of cholesterol to coprostanol by gas chromatography. Faecal tryptic activity was analysed spectrophotometrically.

Results: The allergic infants had lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid. In contrast, they had higher levels of the rarely detected i-caproic acid. This acid has been associated with the presence of *Clostridium difficile*. Furthermore, the allergic infants had higher relative distribution of acetic and i-caproic acid, while the non-allergic infants had higher relative distribution of propionic and valeric acid and tended to have a higher relative distribution of i-butyric and i-valeric acid. None of the other parameters differed between the groups.

Conclusion: The results demonstrate differences in the MACs between allergic and non-allergic infants, indicating differences in the composition of the gut flora. The gut flora may represent an up till now not studied environmental factor delaying the development of a normal Th1-/Th2-balance in allergic children.

## 7:5 Suphasalazine and 5-aminosalicylic acid induce trophic reaction in the gastrointestinal mucosa of germfree rats

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Infammatory bowel disease have been effectively treated with sulphasalazine (SASP) and 5-amiosalicylic acid(5-ASA) but the exact mechanism of their action are hitherto unknown. Earlier studies have shown that SASP, olsalazine and sulphapyridne have a selective compartment dependent proliferative action on the epithelium of the intestinal tract of conventional rats. The aim of this study was to investigate the influence of SASP and 5-ASA on the cell proliferation of the gastrointestinal mucosa of GF rats. For this purpose groups of GF male rats were treated with SASP and 5-ASA and the control rats treated with Placebo. Specimens were collected from different parts of the intestine and colon after injection of metaphase blocker. They were fixed and embedded with liquid paraffin. Results: the percentage of mitotic index (MI%) in the cecum of GF rats treated with both SASP and 5-ASA was increased ($p < 0.05$ GF vs control). The total number of crypt and villus cells in the jejunum and ileum of GF rats treated with SASP and 5-ASA were increased ($p < 0.05$ GF vs control). This study suggest that SASP and 5-ASA have trophic action in the Gl mucosa of GF rats and this action is not influenced by the microflora present in the gastrointestinal tract.

## 8:1 Prebiotics, probiotics, antibiotics & autobiotics

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Probiotics (microorganims) and prebiotics (non-digestible food/feed carbohydrates) have been utilized for centuries in maintaining a balanced gastro-intestinal (GI) microflora. During the last 60 years antibiotics were used extensively as growth promoters. Autobiotic compounds have been identified as important factors in tumor formation some of them are present in the GI tract. Attempts to identify and substantiate the extent of beneficial effect of probiotics has produced data supporting the administration of some lactobacilli and/or bifidobacteria. However, studies with prebiotics were nor as successful. The experience of the continuous utilization of antibiotics has been controversial, to say the least. Relevant information points to the fact that in the long run the price of such practice could be the appearance of more multi-resistant pathogens. Strategies of gut microflora enumeration/identification, effect of prebiotics on probiotic growth and clinical studies have been, and still are the main tools to assess and attempt to understand the action of antibiotics, pro- and prebiotics. Gnotobiology offers more specific, controlled and accurate methods for evaluation of such activities. Germ-free associated characteristics and microflora associated characteristics (MACs) have been studied extensible. MACs are similar in hu-

mans and animals. Therefore, they offer a good basic platform and less variability for the assessment of activities of the microflora and the interaction with prebiotics, antibiotics and autobiotics.

## 8:2 Prebiotics and probiotics; safety aspects and risk assessment

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Many of the prebiotics currently in use are also present in natural foods and may therefore be considered as safe. The consumption of amounts larger then present in foods may lead to changes in the intestinal microflora. These changes should not lead to overgrowth in the intestine of one or a few species. Nor should it stimulate the growth of potentially pathogenic micro-organisms. Lactic acid bacteria (LAB) are ubiquitous inhabitants of the human gastro-intestinal and uro-genitial tract, they are also widely distributed in the environment and in many foods. LAB exert antagonistic activity against many micro-organisms. This property has been exploited in fermented foods. Due to the long history of safe use, especially members of the genus *Lactobacillus* are generally regarded as safe. However, with the introduction of new strains, concern can been raised to their safety in probiotic foods and especially their potential involvement in infections in immuno-compromised subjects. We have studied the potential virulence factors of LAB from bacteremia patients. No common virulence factors could be identified. Though many *Lactobacillus* strains are inherently vancomycin resistant, this resistance does not appear to be transferred. Among the bacteremia LAB isolates no commercial could strain identified. The reported incidence of LAB bacteremia is very low; 0.1–0.2% and patients usually had severe underlying diseases. The wide spread consumption and long history of safe use of LAB and many prebiotics in foods, current preparations and cultures can be considered safe for human use. However, novel prebiotics, probiotic strains and genetically modified strains should be assessed separately for their safety properties.

## 8:3 *In vitro* screening tests for biological activity of probiotics

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Within the last years an increasing amount of products containing probiotic microorganisms, mainly lactobacilli and bifdobactena, were introduced to the market. Most of these products are fermented milk-based products, which have a typically limited shelf life at refrigerated temperatures. Due to the success of these products, interest is increasing to extend the probiotic concept to new product applications and new product categories. Beside the development of closely related produts such as fermented cereal- or vegetable based products, the extension of the probiotic concept to shelf stable products such as milk powder and instant drinks is of high intrest. From a scientific but also from a legal point of view each new product concept requires careful evaluation of the efficacy of the probiotic strains in the new food rnatrix. For closely related products, bio equivalence to the reference product may be demonstrated by comparison of basic process characteristics, complemented with information related to the effective dose and the survival of the probiotic strain during storage. Products being significantly different from the accepted reference require detailed evaluation of the probiotic functionality in the new food matrix. A significant difference consists for example in the physiological state of the probiotic strain in freshly fermented and shelf stable products. In the latter product category the probiotic strain is stabilised in a more or less dehydrated, physiologically completely inactive form. In consequence, it is obvious that the efficacy of the probiotic strain after reconstitution of the dried product requires careful evaluation. It was the objective of our study to investigate the biological activity of the NESTLÈ probiotic strain *Lactobacillus johnsonii* La1 (NCC533) in shelf stable produces. A range of *in vitro* assays was applied allowing us to directly assess properties important for the functionality of *L johnsonii* La1. By means of these *in vitro* assays, factors strongly influencing the functionality of *L. johnsonii* La1 were identified. The application of the acquired knowledge allowed to develop shelf stable *L. johnsonii* La1 preparations with biological activities equivalent to fresh cultures or fermented products.

## 8:4 Use of molecular tools to detect probiotic strains

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A tremendous number of healthful effects have been attributed to probiotics. Traditionally, the strategy developed for detecting and quantifying necessitated two steps. The first one was performed by culturing faecal samples on selective media. The second one was performed by detecting the presence of a given strain among others sharing comparable properties. When used in this second step, conventional methods were time-consuming and often leaded to ambiguous results. The development of molecular methods based on phenotypic or genotypic markers (plasmid profiling, antigen recognition, PFGE, RAPD, colony hybridisation with specific probes, ribotyping, etc …) improved the confidence of the results but were still laborious. The development of molecular sequence analysis, especially of rRNA, provided microbiologists with a powerful tool. The 16S rRNA is an ubiquitary molecule. Its structure, constituted with highly conserved sequences altering with hyper-variable regions, allows to design oligonucleotide probes with variable levels of specificity. These probes can be used as probes in colony hybridisation experiments or as primers to perform amplifications on whole cells. However, the major improvement brought by the knowledge of 16S rRNA sequences is that a specific strain can be detected and quantified by-passing the culture step. Amplification of total RNA and relative quantification can be performed on previously stored samples; techniques based on denaturing electrophoresis (SSCP, DGGE, TGGE) allow a rapid study of the diversity. Finally, 16S rRNA fluorescent probes can be used in automated microscopically-based detection methods. The development of *in situ* hybridisation methods is under progress. Soon, the ecologist will be able not only to specifically detect his probiotic strain, but also to understand its privileged relationships with the host and the resident flora.

## 8:6 Intestinal colonization with a probiotic strain, *Lactobacillus casei* strain Shirota, enhances growth and feed efficiency in a novel gnotobiotic rat model

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To examine the effects of a probiotic strain on the energy metabolism of host animals, we inoculated *Lactobacillus casei* Shirota into gnotobiotic rats (LC-rats). The intestinal physiological and immunological characteristics of our gnotobiotic rats (control rats) associated with rat-derived segmented filamentous bacteria resembled that of conventional ones but their intestinal environmental factors were still simplified. LC-rats had higher

body weight than control rats. The weight gain from day 62 to 118 in LC-rats was significantly larger than that of control rats ($119.0 \pm 7.9$ vs $94.0 \pm 5.5$ g/period, respectively, $P < 0.01$). The growth curve of rats given 3 mM L-lactic acid as drinking water (LA-rats), was not significantly different from that of control rats. Plasma glucose, serum triglycerides and plasma insulin were not different among three groups. However, plasma leptin concentration in both LC- and LA-rats were significantly higher than that of control rats ($13.1 \pm 1.6$, $12.8 \pm 1.3$, $8.2 \pm 1.7$ ng/ml, respectively, $P < 0.05$), suggesting a difference in the energy balance between both the LC- and LA-rats and the control rats. Moreover, feed efficiency (g body weight gain/g food intake) in LC-rats was increased compared with control rats. Colonization with *L. casei* Shirota was suggested to lead to a growth-promoting effect in addition to the contribution of its product, L-lactic acid, to energy metabolism in the host animal.

## 9:1 Overview of mammalian antimicrobial peptides

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Endogenous antibiotic peptides comprise a widespread effector arm of the innate immune system. They have been described in plants, tunicates, insects, fish, amphibia and mammals, and are proposed to participate in the early host defense response against microorganisms. In mammals, their cellular origin includes granulocytes, platelets, specialized epithelial glands, and wet mucosal epithelia of several organ systems. Antimicrobial peptides, which now number greater than 100, can be classified based on structural features. A major focus of our studies has been on α- and β-defensins, peptides with both antibacterial and antifungal activity. We previously characterized from the tracheal mucosa of the cattle the first β-defensin, which was named tracheal antimicrobial peptide (TAP). The TAP gene is expressed in columnar cells of the ciliated respiratory epithelium and expression is dramatically induced by bacterial lipopolysaccharide via a CD14-dependent pathway. Other β-defensins have been since characterized in other organ systems and in other mammals, including humans. A second focus of our studies is on α-defensin expressed in Paneth cells of the small intestine. Here, expression appears to be regulated by post-translational, in addition to transcriptional mechanisms. A current hypothesis is that epithelial expression of defensins contributes to host defense of mucosal tissue. A more complete understanding of these endogenous antimicrobial peptides may lead to the development of useful therapeutics.

Supported by NIH AI32234 and AI32738.

## 9:2 Differential roles of segmented filamentous bacteria and clostridia in the development of the intestinal immune system

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Microflora promotes the development of the intestinal immune system. To evaluate the roles of two types of the indigenous microbes, segmented filamentous bacteria (SFB) and clostridia, whose habitats are the small and large intestine, respectively, in this immunological development, we analyzed three kinds of gnotobiotic mice, contaminated with SFB, clostridia, and both SFB and clostridia, respectively. In the small intestine, the number of TCR-IEL only increased in SFB-associated mice (SFB-mice), but not in clostridia-associated mice (Clost-mice). There was no great difference in V usage among germ-free (GF), conventionalized (Cvd) and these gnotobiotic mice. The expression of MHC class II molecules on the epithelial cells was only observed in SFB-mice, but not in Clost-mice. On the other hand, in the large intestine the ratio of the number of $CD4^-CD8^+$ cells to that of $CD4^+CD8^-$ cells in IEL only increased in Clost-mice, but not in SFB-mice. On association with both SFB and clostridia, the numbers and phenotypes in IEL of the small and large intestine changed to become similar to those in Cvd mice. The number of IgA-producing cells in the lamina propria was more elevated in SFB-mice than in Clost-mice, not only in the ileum but also in the colon. The number of IgA-producing cells in the colon of Clost-mice was a little increased compared to in GF mice. Taken together, these results suggest the presence of the tissue-specific interactions between the indigenous microbes and the host in both the small and large intestine.

## 9:3 Protective effect of a probiotic triassociation—*Lactobacillus acidophilus*, *Saccharomyces boulardii*, *Escherichia coli*—against *Shigella flexneri* and *Salmonella enteritidis* challenge in gnotobiotic and conventional mice

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*Lactobacillus acidophilus* UFV-H2B20 (Gram positive bacteria), *Saccharomyces boulardii* (yeast) and *Escherichia coli* EMO (Gram negative bacteria) are probiotics usually associated with protection of the host against infectious agents of the gastrointestinal tract. In order to evaluate if an unusual combination of the three microorganisms could add the specific mechanisms of protection attributed individually to each of these probiotics, we orally administered *L. acidophilus*, *S. boulardii* and *E. coli* (experimental group) or not (control group) to gnotobiotic (GN) and conventional (CV) NIH mice 10 days before the challenge with two strains of *Shigella flexneri* (a streptomycin resistant mutant designed Sfm and its sensitive wild type Sfw) and *Salmonella enteritidis*. We concluded that the three probiotics used together have a protective effect as demonstrated by: (1) an increased survival of experimental GN mice challenged with *S. enteritidis*; (2) lower histological damage in experimental group of CV mice challenged with *S. enteritidis*; (3) an absence of weight loss in experimental GN mice challenged with *S. flexneri*; and (4) a lower translocation to the spleen in the experimental GN animals challenged with *S. enteritidis*. Moreover, some possible mechanisms of action were investigated and we concluded that: (a) the probiotics inhibited the intestinal colonization of GN mice by Sfm; (b) an antagonistic substance against Sfm was produced only *in vivo* by *E. coli*; and (c) the three probiotics increased the level of IgA secreted in the intestinal tract of GN mice.

## 9:4 Impact of stress protein production on the viability, stability and potential activity of probiotic bacteria

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The stresses associated with the manufacture and storage of lactic acid bacteria (LAB) culture concentrated intended for fermenta-

tion and probiotic applications can dramatically reduce their viability, stability and activity. LAB, like all bacteria, have evolved complex stress responses which are employed to increase the likelihood of surviving adverse environmental conditions. These adverse conditions (stresses) vary with production requirements and include pH shock (fermentation), osmotic shock (drying) and thermal shock (freeze-drying or spray-drying). Expsure to these stresses has been shown to provide protection to lethal levels of the same stress. We are investigating the role of stress proteins on the physiology of LAB and *Bifidobacteria*. Numerous stress-response genes have been characterized in LAB, including those encoding the two major chaperone machines involved with the proper folding of newlly synthesized proteins and the repair of those that are thermally denatured. These chaperones are induced by heat and other stresses and are encoded by the *groESL* and *hrcA*/*grpE*/*dnaK*/*dnaJ* operons. We are currently characterizing the activity of these and other components of the stress machinery of LAB and *Bifidobacteria* strains. The physiological activity of the *groE* and *dnaK* chaperone machines was evaluated during the normal growth cycle and during various stress treaments. As expected, both operons were found to be strongly induced by elevated temperature. The physiological impact of heat shock and other stresses on the viability and storage stability of LAB and *Bifidobaceria* culture concentrates, as well as the genetic basis for these effects, continues to be investigated.

## 9:6 Clinical aspects of synbiotics

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Nosocomial infections constitute an unsolved obstacle to successful surgery especially in elderly and in immune-depressed patients In addition antibiotic resistance is emerging as a new threat. Vancomycin-resistant *Enterococcus faecium* is today in many intensive care units the second to *E. coli* most common cause of bacteremia episodes. The mortality in ICU bacteraemia has remained high (about 40%) over many years, and is no different in *E. faecium* caused infections. Also the rate other complications and sequelae such as thrombosis and adhesion formation remains high and unchanged over the years. It has been suggested that the reduced resistance to disease and particularly infections is related to Western life style and particularly to Western food habits. About 20% of Western population (up to 50% of hospital patients) suffer from Western environmental disease/metabolic syndrome X. It has been speculated that these individuals suffer from an exaggerated acute phase response (APR), manifesting itself in abnormal cytokine production, especially of IL-6. Eighty percent of the human immune system is said to be located in the gastrointestinal tract. Thus it should be possible to nutritionally modulate the APR in connection with disease, trauma and surgery. Lactic acid bacteria and prebiotic fibers can be expected to provide strong such tools.

There is in the literature fast accumulating evidence that probiotic bacteria can control various enteric pathogens such as *Salmonella typhimurium*, *Shigella*, *Clostridium difficile*, *Campylobacter jenjuni*, *and Escherichia coli*, and also various urogenital pathogens such as *Gardnerella vaginalis*, *Bacteroides bivius*, *Candida albicans* and *Chlamydia trachomatis*. WHO experts recommended in 1994 that in addition to dramatic reduction in the use of antibiotics also older forms of therapy such as microbial interference. This far accumulating evidence from the literature and own studies support the use of synbiotics to prevent and control infections, but also to prevent or at least delay the onset of other APR-associated diseases such as arteriosclerosis, cancer, diabetes, neuro-degenerative disease and rheumatoid arthritis. The experimental and clinical experience this far will be reviewed.

## 10:2 Antibiotics and its effects on the host

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An ideal antibiotic exhibits selective toxicity, i.e. it interferes, at concentrations tolerated by the host, with some metabolic and/or other processes that exists only in the parasite and not in the host. However, the ideal antibiotic has yet to be found.

All agents used in human or veterinary medicine or used as food additives will, directly or indirectly, influence upon the host-and his flora. Such host-related influences have to be taken into considerations when strategies for prudent use of antibiotics are under consideration.

## 11:2 The influence of intestinal bacteria on anti-tumor effects of OK-432 in an ascitis model

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A bacterial immunopotentiator OK-432 has been used for malignant diseases and has produced successful results. We investigated how intestinal bacteria influenced anti-tumor effects of OK-432. 84 of specific pathogen free(SPF) BALB/c mice were intraperitoneally inplanted BAMC-1(ascitis tumor model). These mice were divided into 4 groups. Mice in each group received oral administration of suspention of Lactobacilus acidophilus, Echericha coli, Streptococcus faecalis or saline every day. And, 39 of germfree mice, 39 of L. acidophillus gnotobiota, 41 of E. coli gnotobiota and 29 of S. faecalis gnotobiota were inplanted BAMC-1. Thus, 8 experimental groups were established. Half of the mice in each group were treated with five consecutive intraperitoneal injection of OK-432 starting on day 2 following the inplantation. Observation period was 60 days after the inplantation. SPF mice administrated any of these bacteria and treated with OK-432 had significantly longer survival than the mice without OK-432 treatment. L. acidophillus gnotobiota and E. coli gnotobiota treated with OK-432 had significantly longer survival than the gnotobiota without OK-432 treatment. However, OK-432 was not effective for S. Faecalis gnotobiota and germfree mice. SPF mice administrated L. acidophillus and treated with OK-432 had significantly longer survival than L. Acidophillus gnotobiota treated with OK-432. These findings suggested that each species of intestinal bacteria had different influences on anti-tumor effects of OK-432. When L. acidophillus was orally administrated to SPF mice, it would enhance anti-tumor effects of OK-432.

## 11:3 The role of probiotic bacteria in colon cancer prevention

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While a myriad of healthful effects have been attributed to the probiotic lactic acid bacteria, perhaps the most controversial remains that of anticancer activity. Reports in the literature, regarding the anticancer effects of lactic acid bacteria, fall into the following categories: *in vitro* studies and *in vivo* studies in laboratory animals; dietary intervention studies in human volunteers and epidemiological studies correlating cancer and certain dietary regimes. However, it must be emphasised that, to date, there is no direct experimental evidence for cancer suppression in humans as a result of consumption of lactic cultures in fermented or unfermented dairy products. However, there is a wealth of indirect evidence, based largely on laboratory studies, in the literature, and this will be summarized in my presentation.

It must also be pointed out that the precise mechanisms by which probiotic bacteria may inhibit colon cancer are presently unknown. However, such mechanisms might include: enhancing the host's immune response; suppressing the growth of intestinal microflora incriminated in producing putative carcinogen(s) and promoters; binding potential carcinogens; producing antitumorigenic or antimutagenic compounds in the colon; alteration of physiological conditions (e.g. pH) in the colon affecting the metabolic activity of intestinal microflora, the action of bile acids, and causing quantitative and/or qualitative alterations in the bile acid degrading bacteria.

Thus, a number of studies indicate that administration of bifidobacteria or lactobacilli alone or with fermentable carbohydrate (defined as a prebiotic) can alter colonic microflora populations and decrease the development of colonic aberrant crypts (very early preneoplastic lesions) and tumors. However, several studies failed to demonstrate that bifidobacteria or lactobacilli administration alters colonic microflora or has effects on the host. At present, the results from the epidemiological studies do not appear to support the results from experimental studies examining lactobacilli and colon cancer prevention. The reason for this is unclear but might be explained by differences in bacterial strains, with the strains being used in the experimental studies surviving better in the gastrointestinal tract than the strains present in fermented dairy products. It should also be emphasized that great care must be exercised in extrapolating the results of *in vitro* and animal studies to the human system.

However, even with these reservations in mind and mindful of the limited number of human studies available, the use of lactic cultures for human cancer suppression is interesting, holds promise and certainly deserves more scrutiny. The latter should involve carefully designed epidemiological studies to corroborate the wealth of experimental studies.

## 11:4 Studies on the role of human intestinal flora in carcinogenesis exploiting human–flora-associated mice

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Intestinal flora is thought to have a critical role in carcinogenesis. The aim of the present study is to address a possible role of the human intestinal flora in carcinogenesis *in vivo*, by exploiting human–flora-associated (HFA) mice. The capacity of human fecal suspensions to activate or inactivate mutagens was determined using the Ames assay and the human fecal suspensions, active in this regard, were orally inoculated into germfree mice to generate HFA mice. The activity of human intestinal flora against mutagens could be transferred into the mice. HFA, germfree, conventionalized and conventional mice were then orally administered 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ), 2-amino-9*H*-pyrido[2,3-*b*]indole (2-amino-alpha-carboline; AAC) and 2-nitrofluorene (NF) and DNA adduct formation was compared as an *in vivo* biomarker of cancer risk. The presence of intestinal flora was essential for the DNA adduct formation after IQ and NF administration, while DNA adducts after AAC treatment were higher in germfree mice than in mice with bacteria in some tissues including colon. These results clearly indicated that the intestinal flora has an active role in DNA adduct formation. The results also demonstrated that the human intestinal flora has different effects to mouse flora on DNA adduct formation *in vivo* as well as *in vitro* metabolic activities against mutagens. Studies using HFA mice might provide much needed information on the role of the human intestinal flora on carcinogenesis *in vivo*.

## 11:6 Effects of intestinal bacteria on the development of colonic neoplasm induced by DMH

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Effects of intestinal microflora on the development of colonic neoplasm induced by 1,2-dimethylhydrazine (DMH) were observed using conventionalized and gnotobiotic mouse models. The incidence of colonic adenoma in germfree mice(IQI/jic) (GF), mice conventionalized after DMH injection (Cvz-post-DMH) and conventionalized mice (Cvz, conventionalized before DMH injection) was 74, 69 and 58%, respectively. However, the adenoma in Cvz was larger than in GF. The incidence of adenoma in mice mono-associated with *Mitsuokella multiacida*, *Clostridium butyricum*, *Bifidobacterium longum*, *Clostridium paraputrificum*, *Escherichia coli* and *Lactobacillus acidophilus* was 68, 68, 63, 50, 50 and 30%, respectively. However, the adenoma in the *Cl. paraputrificum* group and the *Cl. butyricum group* was larger than in GF. Fecal pH in Cvz and the *L. acidophilus* group was significantly lower than in GF. The deconjugation rate of fecal bile acids in Cvz, the *Cl. paraputrificum* group and the *Cl. butyricum* group was significantly higher than in GF. These findings suggested two different effects of microflora on the development of DMH-induced adenoma, either inhibiting the incidence or promoting the tumor growth. Effects of *L. acidophilus* may be mediated by fecal pH and effects of *Cl. paraputrificum* and *Cl. butyricum* by deconjugated bile acids.

## 11:7 Culture condensate of Bifidobacterium longum as biogenics: Prevention of bacterial translocation and colorectal tumors

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Functional foods related to bacteria are divided into three categories; probiotics, prebiotics and biogenics. Bifidobacterium is one of the most popular probiotic bacteria and many prebiotics are selected on the basis of whether they can increase the number of bifidobacteria in the intestine. However, control of viable bacteria in the intestine is sometimes difficult, especially under pathological conditions. Therefore, we examined the action of culture condensate of B. longum (MB), as biogenics, on physical functions of the host. Feeding of MB diet inhibited bacterial translocation from the intestinal tract to the mesenteric lymph nodes in antibiotic-decontaminated SPF mice and germfree mice, even when the mice were injected with zymosan. In mice fed a MB diet, the CD4 + / CD8 + ratio in the spleen and Peyer's patches (PP) and phagocytic and bactericidal activities of macrophages in PP both increased. Furthermore, feeding of the MB diet reduced the tumor score induced by 1,2-dimethylhydrazine in transgenic mice harboring human prototype c-Ha-ras genes. Bifidobacterium culture is useful for stimulating the defense mechanism as a biogenic functional food.

## 14:1 Prevention of xenotransplant-associated infections

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Largely because of immunosuppressive treatment of the recipient, prescribed to prevent rejection, infections remain a major cause of morbidity and mortality following organ transplantation. These infections can be caused by organisms belonging to the recipient's own microflora, by environmental organisms, and by organisms that are being transferred with the grafted organ. In case of xeno-transplantation, the organisms that can be transferred by the grafted organ are a major concern, since they can introduce 'new' diseases into the human population. Beside viruses and bacteria, many parasites are known to be infective in humans, baboons and pigs. Most ideal would be the use of germfree or SPF donor animals. Pigs have been derived germfree and there is enough gnotobiotic experience to establish a germfree pig colony. Although some experience has been obtained in deriving germfree non-human primates (rhesus monkeys and chimpanzee) this option is not realistic due to the long gestation period and the single offspring they generally produce. Therefore, SPF rearing in a closed colony of non-human primates that are screened routinely for selected viruses might be a more realistic approach. Also a routine screening protocol for the human caretakers should be installed. Wild-caught animals are not acceptable as xenotransplant donors.

## 14:2 Natural antibodies and xenotransplantation

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Transplantation of organs between widely disparate species results in destruction of the graft within minutes to a few hours. This hyperacute rejection process is usually caused by natural (preformed) antibodies in the recipients serum attaching to antigens on the graft endothelium, followed by fixation of complement. Natural xenoreactive antibodies are mainly of IgM-type and are directed against carbohydrate epitopes. Pig is today considered the most likely source animal for clinical xenotransplantation. The major antigen on pig endothelium recognized by human xenoreactive antibodies is the galactose $\alpha$1,3 galactose antigen (Gal-ag). More than 90% of the natural anti-pig antibodies in humans are directed against this epitope. Only man, apes and Old World monkeys lack the Gal-ag and have anti-Gal antibodies while, all other species express the Gal-ag on their endothelium.

Hyperacute rejection can be prevented by removing or regulating either of the three factors; the antigen, the antibodies or the complement. Natural xenorective antibodies occur without previous exposure to the donor species. It has been suggested that natural antibodies arise after exposure to gut microorganisms expressing e.g. Gal. Gnotobiotic animals would then provide a model of natural antibody depletion. However, other studies indicate that many natural IgM antibodies arise early in life and independently of antigenic stimulation.

New-born baboons have only minimal levels of xenoreactive IgM antibodies and have been used as recipients in experimental studies on porcine heart transplantation. In this model, hyperacute rejection does not develop and studies on the later phases of the immune response are then made possible.

For pig-to-human xenotransplantation, other methods to avoid hyperacute rejection are obviously necessary. The development of transgenic pigs expressing human complement regulatory proteins on their endothelial cells seems to offer possibilities to control hyperacute rejection without major interventions in the human recipient.

## 14:3 Mitigation of graft-versus-host disease after allogeneic bone marrow transplantation by modulation of the gastro-intestinal flora of the recipient

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An important factor that influences graft-versus-host disease (GvHD) after allogeneic bone marrow transplantation is the recipient's gastrointestinal microflora. Recipient mice (C3H/Law) carrying a defined rnicroflora (HF) were housed under gnotobiotic circumstances. After total body irradiation (9 Gy, X-rays) they received a bone marrow graft from C57BI/Rij donors, which had either the same flora, or another defined (SPF) flora. The results showed that lethal GvHD was only observed when the recipient harboured microorganisms that were absent in the donor. Complete gastrointestinal decontamination (GID) of these recipients prevented lethal GvHD, in contrast to selective GID that leaves the anaerobic microflora unaffected.

These studies show that GvHD can be induced by activated T-lymphocytes from donor origin reacting against bacterial antigens, which might be cross-reactive with the recpient's epithelial tissue antigens. Activation of these T-lymphocytes is confined to antigens of certain bacteria of the recpient, which are not present in the indigenous microflora of the donor mice. These bacteria most likely belong to the anaerobic flora of the recipient. This hypothesis is supported by the observation in human paediatric patients that, in contrast to complete GID, selective GID did not exert any benefical effect on GvHD after transplantation with MHC-matched sibling donor bone marrow grafts.

## 14:5 Bioclean room and bone marrow transplantation Cost and efficacy

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We have conducted a cooperative study on allogeneic bone marrow transplantation from matched sibling donor for the patients with early leukemia. The study was participated by members from Beijing, Taipei, Hong Kong, Japan, Korea and Malaysia. Bioclean rooms were used in all countries and areas except Hong Kong. The length of clean room treatment varied from 27–66.7 days. Length of hospitalization ranged from 38.6–130.5 days, and 4 months medical costs ranged US$10300–80803. Because the fee for the clean room was one of the major costs in BMT, further studies were performed to reduce the cost. Two standards of clean nursing, strictly clean A level and less strict B level were tested. The number of bacterias was higher in B level, however, there was no difference in the incidence of infection in the patients of matched sibling BMT, while significant difference was observed in patients who received matched unrelated BMT.

## 14:6 Stimulatory effect of Gram-Positive and Gram-Negative probiotics on the host mono-nuclear phagocyte system in gnotobiotic mice

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We studied the influence of Gram-Positive- and Gram-Negative-

based probiotics using Bifidobacterium or non-pathogenic *Escherichia coli* strains on the host's Mononuclear Phagocyte System (MPS) activity of gnotobiotic mice. All probiotics were well established in gnotobiotic mice achieving $10^9$–$10^{10}$ viable cells per gram of faeces by days 7–11. The growth of *E. coli* in combination with *B. bifidum* was lower than in monoassociation conditions. The number of small intestine intraepithelial lymphocytes in probiotic-associated mice slightly increased in comparison with germfree mice, although it did not reach the number seen in conventional animals. The clearance capacity of gnotobiotes against *E. coli* $B_{41}$ and *S. typhimurium* was significantly higher than in control germfree animals and was correlated with an increase in Kupffer cell's number. Preopsonization of the test-pathogen with blood sera from probiotic-associated mice led to some increase in *Salmonella typhimurium* clearance in conventional mice which, however, was lower than that produced by conventional sera. These results show the direct stimulatory effect of probiotics on the host's MPS (through both cellular and humoral mechanisms) and indicate that the combination of anaerobic and aerobic probiotics may be beneficial.

Supported by FAPEMIG and CNPq.

## 15:3 The influence of intestinal flora on the healing of intestinal anastomosis in rats

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This study attempted to elucidate the influence of the intestinal flora on the healing process in intestinal anastomoses. Five groups of rats were studied, consisting of germfree, conventional, mono-contaminated with Lactobacillus acidophilus or Escherichia coli and ex-germfree rats. All rats underwent ileal and colonic resections followed by anastomoses. Seven days afterwards, they were sacrificed, and the bursting pressure and hydroxyproline concentration of the anastomoses were measured. The microbiological status of the animals was confirmed weekly. No bacteria in the germfree rats or any other bacteria in the mono-contaminated rats were detected. Conventional rats had a significantly higher anastomotic bursting pressure than either mono-contaminated rats with L. acidophilus in the ileum, or germfree rats in the colon. The ex-germfree rats also showed a significantly higher bursting pressure than the germfree and rats mono-contaminated with either L. acidophilus or E. coli in the ileum and colon. The presence of the intestinal flora enhanced the healing of intestinal anastomoses. The data suggest that this effect depends on differences in the types of bacteria in the intestine.

## 15:4 The intestinal flora and postsurgical adhesion formation

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The literature is rich in conflicting reports on the efficacy of different treatments for postsurgical adhesion formation. Development is hampered by the fact that the pathogenesis is not fully elucidated. In this study germfree rats were either turned into exgermfree rats by faecal flora enemas, thereby establishing a common bowel flora, or subjected to dicontamination with *E. coli* and *B. fragilis*. Experimental adhesion formation was performed and it was found that exgermfree rats (82.5%) have the same adhesion forming ability as conventional rats (87.5%) and differ from germfree rats (20%) by the same magnitude, whereas dicontaminated rats (50%) fall in between. In conclusion, exgermfree rats form adhesions as readily as conventional ones, and this further indicates a role of the native bowel micro flora in adhesion formation, although it cannot solely be attributable to *E. coli* and *B. fragilis*.

## 15:5 A model for foreign body prophylaxis and treatment in ex-germfree rats

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Foreign body infections are an important problem in modern medicine. Few animal models exist that allow study of prevention and therapy of these infections. We developed a subcutaneous catheter model in the rat that allows study of the prevention and treatment of foreign body infections by coagulase negative staphylococci.

A commercially available catheter segment is incubated with a defined low inoculum of *Staphylococcus epidermidis* just prior to implantation. In normal Fisher rats or in inbred Fisher rats, the infecting bacteria are rapidly eliminated in most of the catheters. In inbred germfree rats and ex-germfree conventionalised rats, a reproducible infection follows in almost 100% of the catheters if no prophylaxis is given. In treatment experiments we found that antibiotics with known activity in foreign body infections in man had an effect on the foreign body infection that was similar to that observed in the human host.

We conclude that the ex-germfree rat model is a valid model for the study of foreign body infection prevention and treatment.

## 15:6 Interaction of *Lactobacillus fermentum* and *Escherichia coli* in guinea pig testes model

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Interaction of probiotic lactobacilli and potentially pathogenic microbes of indigenous micro-flora has usually been investigated *in vitro*, and only rarely in tissues of experimental animals. However, the possible translocation of mucosal microbes has risen interest to these experiments.

The aim of the study was to elucidate whether *Lactobacillus fermentum* are able to suppress the multiplication of *Escherichia coli* and prevent the development of tissue alterations in an infection model of guinea pig testes.

Material and methods: Altogether 48 adult guinea pigs were inoculated: Group I—animals with a suspension of clinical isolate of *Escherichia coli*; Group II—human *Lactobacillus fermentum*; Group III—*L. fermentum* + *E. coli* simultaneously; GroupIV—*L. fermentum* 24 h before adding *E. coli*; 3 guinea pigs served as control. The animals were killed at different intervals (1 and 6 h, 1, 2, 3 and 5 days). The blood, liver, spleen and testes samples were cultured on MRS and Endo media; the aforementioned organs were histologically examined in preparations, stained by hematoxylin-eosin and the modified Gram method.

Results: In blood of Gr I and Gr II animals the *L. fermentum* and *E. coli* were revealed 6 h and 24 h after inoculation and in testes their maximal counts (6.9 and 8.6 log/g, respectively) were detected after 6 h. Lactobacilli induced proliferative whereas *E. coli* exudative inflammatory reaction, mainly with multiple granulocytes. In Gr III the pathological changes were similar to these of previously described, yet even more intense. In contrast, the preceding inoculation of *L. fermentum* (Gr IV) suppressed the

counts of *E. coli* (nearly for 3 log) and decreased both the proliferative and exudative inflammation in testes. In blood samples the *E. coli* was found only 6 h after inoculation.

Conclusion: Previous inoculation of *L. fermentum* can suppress both the growth of *E. coli* and inflammatory reactions, induced by the bacteria.

## 15:7 Selective translocation of coliform bacteria adhering to caecal epithelium of rats during catabolic stress

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Translocation from the intestine to the blood stream of coliform bacteria in patients under severe stress may result in gramnegative septicaemia and constitutes a clinical problem. The mechanisms for this translocation is unknown. We have developed a catabolic stress rat model using starvation and/or controlled bleeding analysing the coliform flora using a biochemical fingerprinting method (The PhenePlate™ system).

Results. It was found that after starvation for 24–48 h the number of intestinal coliform bacteria increased 100–1000 fold. Certain strains adhered strongly to the intestinal wall, and among these a few strains were able to translocate to the mesenteric lymph nodes (MLNs). In rats spontaneously lacking such strains translocation was not possible to induce, but this was reversed through colonisation with such strains. Similarly, selectively translocating strains were detected also in pigs and in a human case. These strains of *E. coli* are considered to represent Translocating *E. coli* (TEC), which may constitute a new group of potentially pathogenic *E. coli*.

## 16:1 Normal luminal bacteria are required for chronic, immune-mediated intestinal inflammation in genetically susceptible rodents, but all endogenous bacteria are not equal

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We have demonstrated in a series of gnotobiotic experiments that Lewis rats injected with indomethacin, HLA B27 transgenic rats, IL-10 and IL-2 deficient mice and $CD_3\varepsilon_{26}$ transgenic mice fail to develop chronic intestinal inflammation and mucosal immune cell activation in a germ free (GF) environment, in striking contrast to the aggressive T lymphocyte-dependent colitis and enteritis that develops in these specific pathogen free (SPF) rodents. Colitis develops in adult GF IL-$10^{-/-}$ mice within 1 week after colonization with SPF bacteria, and is attenuated by antibiotics, particularly those with broad spectra of activities. Transfer of mesenteric lymph node (MLN) cells from SPF TG$\varepsilon_{26}$ TG mice with colitis to GF recipients caused no disease. However, transfer of MLN cells from GF donors to SPF recipients induced colitis within 4–6 weeks, indicating that constant exposure to luminal bacterial antigens is necessary to cause colitis. CD4+ MLN cells secreted IFN$\gamma$ in response to APCs pulsed with luminal bacterial sonicates, but not non-bacterial antigens. We demonstrated in selective reconstitution and monoassociation experiments that *Bacteroides vulgatus* was preferentially able to induce colitis in HLA $B_{27}$ TG rats, but that *Escherichia coli* monoassociation was no different from the GF environment. However, this same *B. vulgatus* strain did not induce aggressive colitis in IL-$10^{-/-}$ mice. *Lactobacillus plantarum* was superior to *Lactobacillus GG* in preventing colitis in gnotobiotic IL-$10^{-/-}$ mice subsequently colonized with SPF bacteria and *L. GG* could attenuate reactivation of colitis in SPF HLA $B_{27}$ TG rats transiently treated with broad spectrum antibiotics. Together, these experiments demonstrate that certain endogenous bacteria preferentially induce immune-mediated colitis in genetically susceptible hosts, and that others are protective. Manipulating the relative balance of these luminal constituents may have therapeutic value in human inflammatory bowel disease.

## 16:2 Xenobiotics, intestinal microflora and innate gastrointestinal immunity: What can clinical observations and experimental animal models tell us about environmentally related diseases

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Xenobiotics refer to any substance, wheter manmade or natural, which is by nature foreign to the human. Xenobiotics can e.g. be immuntoxic depending on molecular modification, exposure routes and time. Carrageenan, a sulfated polysaccharide which is the natural product of red sea algae, is extensively used as a stabilizer in human food. When modified regarding molecular weight and sulfation it becomes a powerful inducer of intestinal inflammation and tumor genesis. This inflammogenic property, shared by other sulfated polysaccharides e.g. DSS, have now on one hand been shown in animals to be independent on the presence of an intestinal microflora. On the other hand, the presence of bacteria modulate and even reverse the inflammation. Control of the small intestinal microflora has been attributed to antibacterial substances, such as e.g. lysozyme, produced by Paneth cells. Recently these cells have also been shown to produce antimicrobial peptides. Such peptides are produced in other organs as well and are believed to play an important part in the innate immunity of the host. Together with e.g. the complement system such mechanisms can engage very early when the host is exposed to microbial contamination and there is little time for an classical immunereaction or the the immune system is still undeveloped. E.g. it has been shown that children with necrotic enterocolitis lack normally developed Paneth cells which could contribute to bacterial overgrowth and malignancy. In an preliminary investigation, adult small intestine subjected to bypass surgery shows abnormal Paneth cell distribution pointing to that the intestinal environment is important in the regulation of Paneth cells.

## 16:3 The role of bacterial microflora in development of dextran sodium sulphate (DDS) induced colitis in immunocompetent and immunodeficient mice

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Immunocompetent Balb/c mice and mice with severe combined immunodeficiency (SCID) reared under conventional and germfree conditions were used to analyse the role of microflora in development of intestinal inflammation. Balb/c and SCID mice reared in conventional conditions (colonized by microflora) developed clinical and histopathological signs of colitis after 7 day feeding with DSS (rectal prolaps, colonic bleeding, epithelial

erosions and ulcers, increase in the number of inflammatory cells in lamina propria and submucosa, etc). The intestinal mucosa of these mice reared in germfree conditions showed only minor changes after DSS feeding. The possible role of breakdown of oral tolerance to microflora antigens in experimentally induced intestinal inflammation was analysed by oral administration of microflora sonicate. We showed that severity of DSS induced intestinal inflammation in Balb/c mice is reduced by orally administered microflora sonicate.

## 16:5 Signaling to NF-$_k$B through the Toll/IL-1 pathway

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Signaling through the Toll/IL-1 receptors utilizes conserved intermediary molecules to activate the transcription factor, NF-$_k$B. A number of intermediary proteins have now been identified and according to the current model engagement of the ligand with the receptor results in recruitment of cytoplasmic protein kinases and adapter molecules. Ultimately the signaling by these molecules leads to activation of a kinase, NIK (NF-$_k$B inducing kinase), which directly activates I$_k$B kinase, leading to phosphorylation and degradation of I$_k$B and activation of NF-$_k$B. We have been interested in further elaborating this pathway and have identified novel adapter molecules that are involved in signal transduction to NF-$_k$B. Characterization of these molecules as intermediates in the Toll/I1L-1 pathway will be presented.

## 16:7 Water extract of *Helicobacter pylori* stimulate the mitotic activity in human explants of gastric mucosa

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*Helicobacter pylori* contain mitogen(s) to epithelial cell lines. Our aim was determine whether a water extract of *H. pylori* affect the mitotic activity in human antral glands and the release of proinflammatory cytokines from the mucosa. Biopsy specimens from the antral mucosa of 12 subjects that underwent upper endoscopy for investigation of dyspeptic symptoms or chronic gastrointestinal bleeding were used. The gastric mucosa had a normal appearance and the histological examination and CLO test excluded gastritis and *H. pylori* infection. The specimens were processed following guidelines for organ culture technique. The explants of gastric mucosa were incubated 24 h with water extracts of *H. Pylori* 88-23 and in the last 3 h vincristine was added to arrest mitoses in the metaphase. The explants were thereafter processed for microdissection. Antral glands were microdissected at light microscopy and the total number of metaphases per crypt was determined. In other explants, the release of TNTa, IL-1, IL-6, IL-8 and IFNi to the culture medium was estimated. The water extracts of *H. pylori* significantly increased the total number of metaphases per crypt compared to controls. The water extract did not affect the release of cytokines to the culture medium. Our findings indicate that mitogen(s) of *H. pylori* increase cell proliferation in human gastric epithelium and that the mitogenic action is not secondarily mediated by cytokines that are commonly synthesized following infection with *H. pylori*.

## 16:8 Helicobacter pylori and gastric carcinogenesis

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Background and Aims: Helicobacter pylori (Hp) is known to cause gastric diseases including carcinoma. This study was performed to investigate into the role of Hp in relation with the gastric carcinogenesis Germ-free C3H mice and it's counterpart of the conventionalized mice. Materials and Methods: Conventional and germ-free C3H male mice, 7 weeks old, were administered N′-methyl-N′-nitrosourea (MNU) ad libitum via drinking water at the concentration of 120, 80, 40 or 0 ppm (freshly prepared every other day) for 20 weeks. Then (at 27 weeks old), about one-third of the MNU-treated germ-free mice were gastrogavased with 109 living H. pylori. Mice of the 3 groups (conventional, germ-free and gnotobiotic) were sacrificed at 47 weeks and subjected to cultivation of gastric contents for Hp and histopathologic examinations of the stomach. Results: Hp infection of the stomach was demonstrated in all Hp-infected mice at autopsy. Gastric neoplasms including adenomatous hyperplasia were more frequently observed in germ-free mice than in conventional ones at 40 and 120 ppm of MNU ($p < 0.05$). However, there was no significant difference in the incidence of gastric neoplasms between germ-free and gnotobiotic groups. Conclusions: Conventional bacterial flora inhibited gastric tumorigenesis. Hp infection did not seem to increase the risk of gastric cancer in this model.

## 16:9 Antibacterial peptides from *Helicobacter pylori*

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Infection by *Helicobacter pylori* is estimated to be the most common bacterial infection in humans and its prevalence in underdeveloped areas may approach 100%. Even though colonisation with this organism is a predisposing factor for several gastro-intestinal illnesses, most infections are asymptomatic implying that *H. pylori* may be considered as part of the normal microflora. As such *H.pylori* may have beneficial effects in infected individuals by contributing to the microbial balance in the gastro-intestinal tract.

We have shown that extracts of *H. pylori* possess an antibacterial activity and traced this activity to the cecropin-like N-terminus of ribosomal protein L1 (RpL1). The activity of these peptides were of the same range and specificity as cecropins. *H-pylori* is known to lyse extensively during *in vivo* growth. We suggest that the peptides are released to the stomach through bacteriolysis. By using a gnotobiotic human-Le b$^+$ transgenic mouse model for colonisation of the stomach by *H. pylori* (in collaboration with Per Falk (Karolinska Hospital, Sweden) and Brita Björkholm (SMI, Sweden)) we will investigate the *in vivo* significance of our finding. Gnotobiotic Le-b$^+$ mice were mono-infected for seven weeks by *H. pylori*. The mice were sacrificed and the stomachs were dissected out. All mice were checked for colonisation of the stomach by viable count and immunohistochemistry. *H. pylori* positive respective negative stomachs were pooled in two separate groups and processed for HPLC analysis for antimicrobial peptides and for immunohistochemistry using a specific anti-*H. pylori* RpL1 peptide-antibody. Data from these experiments will be presented in a poster.

## 16:11 The small intestine of germ-free mice contains antibacterial factors: a bacterial monocontamination induces additional antibacterial peptides, a response that is blocked by a cortisone pretreatment

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Background: Germ-free (GF) mice are useful to compare innate and immune responses to microbes. Some effector molecules of innate

immunity are gene-encoded antimicrobial peptides. Mice are known to produce at least 17 enteric antimicrobial peptides, i.e. defensins. Frog peptides can eliminate an infection with *Aeromonas hydrophila*, strain Bo-3, but pretreatment with cortisone block these peptide genes via NFkB/IkBa.

Aims: To investigate if the sterile intestine of GF mice contained antimicrobial factors and if bacterial monocontamination would induce additional factors. We also wanted to test if mouse peptide genes were blocked by pretreatment with cortisone.

Methods: Mice received strain Bo-3 (dose 109 CFU) by intragastric gavage and were killed after 4, 8 and 24 hours. Additional mice were given i.p. cortisone for 3 days prior to this monocontamination. The small intestine was collected, quickly frozen in liquid nitrogen and extracted with TFA and acetonitrile, freeze dried and redissolved in water. It was analysed by HPLC using a C18 column and elution with an acetonitrile gradient. The fractions were analysed for antibacterial activity as inhibition zones on agar plates and transformed to cecropin peptide units by a standard curve.

Results: A single HPLC fractionation of the crude extracts showed that the sterile intestine of GF mice contained tree antibacterial components (the two last poorly resolved). An monocontamination with *Aeromonas hydrophila* produces two additional components. A pretreatment with cortisone abolished the two first peaks with antibacterial activity.

Conclusions: GF mice that have never been exposed to bacteria produce antimicrobial peptides. After a monocontamination two additional component were produced. The response is fast, occurring within hours after challenge. The intestinal response induced by an monocontamination was blocked by systemic cortisone pretreatment which implies a gene control by NFkB/IkBa.

## Antagonistic activity of lactobacilli against urinary *Eschericia coli*

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The intestinal tract has been considered previously as a reservoir for *Escherichia coli* strains in recurrent urinary tract infections (UTI). To control colonisation of these *E. coli* strains the administration of lactobacilli (LAB) has been suggested. The aim of the study was to investigate if different LAB strains inhibit UTI *E. coli in vitro*.

Materials and methods: UTI *E. coli* strains (19) isolated from 14 children with recurrent UTI were tested against 6 intestinal LAB strains, selected from healthy Estonian and Swedish children and 2 commercial probiotics (GEFILUS, LINEX). The bactericidal and bacteriostatic activity of lactobacilli against *E. coli* was estimated using modified MRS broth and MRS agar (pH 7.2).

Results: Based on the bactericidal effect exerted by LAB versus *E. coli*, the LAB could be divided into 2 groups. Group I lactobacilli (*L. paracasei*, *L. plantarum*, *L. Rhamnosus* [GG]) decreased *E. coli* counts from control levels, $10^6$–$10^7$ CFU/ml, to $0–5 \times 10^3$ CFU/ml, whereas group II lactobacilli (*L. plantarum*, *L. fermentum*, *L. buchneri*, LINEX) decreased counts to $5 \times 10^4$ $10^6$ ($P < 0.001$). This bactericidal effect was associated with pH values measured after co-cultivation (group I median 3.65, group II median 3.92, $P < 0.001$). No correlation between bacteriostatic and bactericidal effects of LAB was found and it was independent of spp., $H_2O_2$ production and anti-oxidative activity of LAB. There were no differences in the bactericidal activity of LAB against *E. coli* strains which had been isolated from patients during the first attack of pyelonephritis or recurrent infection thereafter.

Conclusion: Certain LAB strains inhibit growth of UTI *E. coli in vitro* and this suppression is dependent on low pH.

## Interaction between commensal bacteria and human intestinal epithellal cells *in vitro*

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The intestinal mucosa, colonised by commensal microorganisms, constitutes the interface with the external environment, through which most pathogens initiate infectious processes. Intestinal mechanisms of defense need to discriminate accurately between commensal microflora and exogenous pathogens. Epithelial cells that line the intestinal tract (IEC) are considered to participate in the initiation and regulation immune responses to bacteria by interacting with immunocompetent cells. We studied the capacity of mucosal immunocompetent cells to react to bacterial signals generated by non-pathogenic microorganisms using a newly developed human *in vitro* co-culture model. A characteristic response to different components of the microflora has been detected. Two different patterns of innate immune response were shown, discriminating between Gram-negative and Gram positive bacteria. Furthermore, differences in the cellular activation of IEC, with respect to expression of pro-inflammatory and regulatory cytokines, by non-pathogenic *Lactobacillus* species were observed. We demonstrate the importance of non-pathogenic microorganisms in modulation of the host mucosal defenses and maintenance of homeostasis and gut integrity. The understanding of these modulatory functions will provide unique opportunity to prevent or treat intestinal disorders associated with food allergy, intestinal infections, inflammatory bowel disease and autoimmunity.

## Monocontamination of germ-free NMRI mice with different probiotic bacterial strains

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Several bacterial strains belonging to the genera Bifidobacteria, Clostridia, Enterococci, Lactobacilli, and Streptococci, have been used as probiotics. Seven of them, *Streptococcus thermophilus* strain ATCC19268, *Lactobacillus delbrueckii* subsp *bulgaricus* DSM 20081, *Bifidobacterium bifidum* B12, *Streptococcus thermophilus* strain B16, *Bifidobacterium bifidum* B11, *Lactobacillus acidophilus* La-5 (Tine) and *Lactobacillus plantarum* strain 299, have been selected to monocontaminate groups of four germ-free NMRI mice in order to test some intestinal biochemical functions using the GAC/MAC concept (Germ-free Animal Characteristics/Microflora Associated Characteristics). After 7–10 days colonization, the following MACs were analysed in colon contents: conversion of cholesterol to porostanol and bilirubin to urobilinogen, degradation of β-aspartyglycine, triptic activity and mucin, and the profile of short-chain fatty acids (SCFAs). All these probiotic strains were able to alter the SCFAs profile significantly as compared to germfree mice. Acetic acid accounted for more than 98% of the total SCFAs concentration in all the groups. Among the seven bacterial strains used as monocontaminants, *Streptococcus thermophilus* induced the highest increase in the total SCFAs concentration. Within the other biochemical functions investigated, smaller alterations in some of the animals were detected.

## The digestive tract of American black vulture (*Coragyps atratus* Bechstein 1793): bacterial microbiota and research for antagonistic substances

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The American black vulture is a bird commonly associated with human activities and widely distributed throughout the American continent. As a carrion-feeding bird, it seems plausible that the vulture digestive tract has potent anti-microbial mechanisms because much of its diet consists of animals that have died of infectious diseases. Although there is speculation about this hypothesis, scientific studies are scant and even the constitution of the digestive microbiota has been overlooked. In the present communication, the isolation, count and identification of aerobic and anaerobic bacteria along the digestive tract of six vultures are reported. After the capture, the birds were submitted to an 1 week period of nutritional adaptation to eliminate possible allochthonous microorganisms. Then, specimens collected from tongue, stomach and intestines were weighed, submitted to decimal dilution in an anaerobic chamber, inoculated on selective and universal culture media and incubated aerobically and anaerobically at 37°C. The microbial population levels ranged from $3.46 \pm 0.39$ log CFU/g in the stomach to $11.94 \pm 0.57$ log CFU/g in the distal intestine. Isolated bacteria were used as producer and revealing strains to detect antagonistic phenomenon. The observed hetero-, iso- and autoantagonism relationships support the potential ecological role that these microorganisms have in terms of population auto-control and environmental barrier, preserving the balance of the gastrointestinal ecosystem of vultures. This ability may be directly involved in and contributing to the remarkable anti-microbial resistance presented by those birds.

## Role of elements of the gut microbiota in driving the normal development of the gut mucosal immune system and in complementing abnormal inflammatory reactions in the gut

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We have been using germ-free (GF) and gnotobiotic mice to try to establish some basic principles concerning the roles of members of the gut microbiota in both normal and abnormal inflammatory reactions in the gut. Our main findings thus far are:

1. that both acute reovirus infection of the gut and chronic colonization of GF mice with Morganella morganii result in transient germinal center reactions (GCR) in Peyers patch (PP) and mesenteric lymph nodes (MLN), which wax and wane, while specific, secreted IgA Abs rise and only decline gradually over many months;

2. certain normal (segmented filamentous bacteria, SFB) and occasional (Listeria monocytogenes) colonizers of the gut stimulate the formerly GF gut to develop near normal levels of natural IgA secreting cells (ASC), while other gut commensals: Helicobacter muridarum, M. morganii, Ochrobactrum anthropi stimulate far lower levels of natural ASC and IgA and relatively more specific Ab secreting cells (AbSC) and IgA Abs, except for O. anthropi, which seems to stimulate minimal aspecific or nonspecific responses;

3. although successive gut infections with reovirus result in successively decreasing GCR in PP and MLN and lesser mucosal IgA responses, the supercolonization of initially SFB colonized, formerly GF mice with M. morganii results in a new set of GCR in PP and MLN and the new appearance of specific IgA Abs to the secondary colonizer;

4. using avirulent mutants of L. monocytogenes (actA-, D-ALA-) we were able to safely colonize the intestines of GF mice and demonstrate a local, specific IgA Ab response. These mice, which are chronically colonized with the actA(-) mutant or which rapidly lose the D-ALA($-$) mutant, are both resistant to oral challenge with virulent, wild-type Listeria;

5. that severe-combined immunodeficient (SCID) mice and immunocompetent (IMCOMP) mice, both GF, can be crossed and back-crossed and used to demonstrate both the effect of maternal IgA Abs and of actively developed IgA Abs to modulate and restrict the whereabouts and numbers of SFB in the neonatal gut;

6. using SCID mice we have tried to identify those members of the gut microbiota that may interact with dysregulated T cells to contribute to the development of inflammatory bowel disease-like symptoms. We have found that transfer of CD4+, CD45RBhigh T cells into GF mice does not result in IBD symptoms, while transfer into conventionally reared (CNV) mice has been found by others to do so. Monoassociation of GF mice with a series of elements of the gut microbiota: M. morganii, O. anthropi, L. monocytogenes actA($-$), SFB do not. Presently we are exploring the possibility that intracellular microbes such as rotavirus EDIM or H. muridarum can generate gut epithelial cell targets for specific or aspecific attack, that may lead to IBD symptoms.

## Probiotics or antibiotics to piglets

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For many years, antibiotics have been used as feed additives to piglets and other domestic animals, and probiotics are now increasingly used. In the present investigation, the antimicrobial substance bacitracin and the probiotic strain Bacillus licheniformis have been added to piglet litters' feed. Their effects upon intestinal metabolism have been measured by following parameters of microflora associated characteristics (MACs): coprostanol, urobilinogen, (-aspartylglycine, tryptic activity and short chain fatty acids (SCFAs). Bacitracin, Bacillus licheniformis and both together were given to litters of piglets at 3 weeks to 10 weeks old, and faecal samples, taken at intervals (3 weeks, 5 weeks, 7 weeks and 10 weeks old), were analysed. At 5 weeks age, piglets given both feed additives had significantly higher total production of SCFAs. No alterations from control values were found in the other MACs. All four groups demonstrated similar increases in weight during the observation period. Conclusively, bacitracin and Bacillus licheniformis had an additive effect upon the SCFA parameter at weaning period.

## MACs for Omnivores and Herbivores

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Animal hosts have 'digestive incubation chambers', e.g. rumen and caecum, in which microbial fermentation proceeds under optimal conditions. Omnivore animals, e.g. humans, have an intestinal microflora adapted to 'fast food', as simple carbohydrates and proteins, to be digested before colon. Each species of animals do likely have their own flora which has evolved with the

host over millions of years. For example, in humans the most common microbe in colon and faeces is Bacteroides vulgatus, but pig flora is dominated by Streptococcus intestinalis. Another way to characterise the intestinal flora of animal species and individuals could be to measure specific metabolic parameters of activity of the flora, namely Microflora Associated Characteristics (MACs). Basic values for chosen MACs should then possibly characterise the 'normal' functional flora on species level.

In three omnivores (humans, rats, pigs) and two herbivores (horses, cows), the following six parameters have been investigation: Short chain fatty acids (SCFAs), urobilinogen, tryptic activity, coprostanol, (-aspartylglycine and mucin.

The basic values in faecal samples for the first three parameters were different between omnivores and herbivores, but the last three were similar. However, no qualitative differences in MACs' pattern were seen in any of the species investigated.

## Microbial functions in ecological and conventional reared pigs

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Pigs and other animals are born germfree, and immediately after birth microbes invade the gastrointestinal tract. Microbiological enumeration and identification studies can today be supplemented with studies on functions related to presence of any microorganism(s) and related host-organism. These functions are termed Microflora-Associated Characteristics (MACs) and the parameters used in this study were mucin, coprostanol, urobilinogen, (-aspartylglycine, tryptic activity and short chain fatty acids (SCFAs). These MACs are used to measure the development of the microbial flora in faecal samples of both ecological (born and reared outdoors) and conventional (born and reared indoors) piglets, from birth to slaughter-age ($\sim$160 days). To that, MACs in samples from healthy mother-sows were investigated as comparable baseline values for adult conventional pigs. This study showed that within some age groups, the MAC-parameters mucin, tryptic activity and urobilinogens were significantly different in faecal samples from piglets born indoors (conventional) and outdoors (ecological), but no difference were seen regarding the coprostanol, (-aspartylglycine and SCFA parameters. Whether—and to what extent—these differences in microbial intestinal functions may influence upon prevention of diarrhoea in the weaning period remain to be investigated.

## Reversibility of oxygen tolerance for oral *Fusobacterium* strains after an adaptive period in anaerobic chamber and digestive ecosystem of gnotobiotic mice

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The genus *Fusobacterium* includes strict anaerobic Gram negative rods found in the normal digestive microbiota but also involved in several clinical infections. This amphibiotic relationships with the host may be related with a variable capacity of aerotolerance permitting the passage from an extremely reduced (digestive ecosystem) to a more oxidated environment (internal host compartment). In the present study, 19 oral strains of *Fusobacterium* recovered from human beings (07) and marmosets (12) were evaluated for their progressive aerotolerant capability soon after their isolation and after an handling period of 2 months in an anaerobic chamber (85% $N_2$, 10% $H_2$, 5% $CO_2$). Inversely, a *Fusobacterium periodonticum* showing a very high aerotolerance (tolerance time: 56 h) after the 2 month handling period was orally administered to gnotobiotic mice and recovered after 5, 10 and 15 days for re-evaluation of its aerotolerance. *Fusobacterium* strains were more aerotolerant (tolerance time ranging from 20 to 56 h) after handling than immediately after isolation (tolerance time ranging from 1 to 9 h). On the other hand, the aerotolerance of *F. periodonticum* decreased drastically from 56 to 10 h after a 5 day association in the digestive tract of the gnotobiotic mice. We concluded that the *Fusobacterium* strains used in these experiments present a reversible aerotolerance probably important for the adaptation of these bacteria to the environment.

## Gnotobiotics and environmental toxicology: Lead (Pb) bioavailability

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The gnotobiotic containment facilities were used to eliminate cross-contamination during 30 days of quantitative feeding of low levels of lead (Pb) in rats and minipigs. Pb acetate (PbAc) and Pb nitrate (PbNO) were fed to control animals at the same concentrations. The results showed: 1) good dose-response of Pb in all organs; 2) significant increases in relative PbNO lead bioavailability in rat bone; 3) Organ depositions of Pb were 8, 8, 4 and 2 times higher in bone, liver, blood and kidney of minipigs, respectively, than in the control rats' organs. In a similar study, the ability of composts/biosolids to bind soil Pb and make it non-absorbable was tested in rats. Four of 7 composts/biosolids tested significantly reduced bone Pb. Finally, the role of melanin in skin uptake of Pb was investigated by *in vitro* incubations of rat skin. The amount of Pb bound and the amount of melanin in the skin were 1.7 and 9.0 times larger respectively in the dark than in the light skin. Thus, environmental toxicology has benefited from the application of gnotobiotic technology.

## The normal microflora influence the immune response to Mycoplasma pulmonis in the nasal mucosa

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Little is known about the role played by the microflora in regulation of immune responses in the upper airways. Here, we report that the normal microflora reduce the numeric and phenotypic changes of lymphocytes in the rat nasal mucosa after monoinfection with *Mycoplasma pulmonis*. Intraepithelial and lamina propria lymphocytes were analysed by paired immunofluorescence staining *in situ* in germ-free and conventional AGUS rats before and at three weeks postinfection. The numbers of both intraepithelial and lamina propria lymphocytes increased slightly more in germ-free than in conventional rats as a result of monoinfection (2.4 times vs. 1.4 times, n.s.). Furthermore, a striking increase was observed in the proportion of CD4 + TCR-$\alpha\beta$ + T cells in germ-free rats, both in the epithelium ($p < 0{,}001$) and the lamina propria ($p < 0{,}001$), whereas this subpopulation showed a modest increase (n.s.) in conventional rats. Conversely,

the CD8 + TCR-αβ + subpopulation was relatively decreased in both categories. TCR-γδ + T cells were scarce in the nasal mucosa (less than 5%) and did not change numerically or phenotypically after infection. The size of NALT (nasal-associated lymphoid tissue) also increased slightly more under germ-free conditions (5.1 times vs. 2.6 times, n.s.). Interestingly, we found a large population of CD8 + lymphocytes that did not coexpress TCR- αβ or TCR-γδ. Many of these cells coexpressed the NK-marker NKR-P1 and were also CD2 + , and thus appeared phenotypically as NK cells. In the conventional category there was a tendency toward a relative decrease of this subset after infection while no difference was observed between the two germ-free groups. Together, these results suggested that the presence of normal microflora attenuate the response of certain T-cell subpopulations in the rat nasal cavity after monoinfection with *Mycoplasma pulmonis*.

## LPS-induced production of cytokine, $PGE_2$ and corticosterone in serum and macrophages from germfree and conventional rats

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To clarify microflora-associated differences in LPS (lipopoly-saccharide) sensitivity, we measured the levels of TNF, $PGE_2$ and corticosterone induced by LPS in serum and thioglycolate-elicited macrophages from germfree and conventional rats. The animals used were 8–10-week-old WA/Jic conventional and germfree male rats, and macrophages were prepared from their rats 4 days after i.p. injection of 3%-thioglycolate medium. LPS-induced elevations of serum TNF and $PGE_2$ levels were significantly less in germfree rats, while the elevation of serum corticosterone was greater in germfree rats than in conventional rats. Macrophages from germfree rats secreted lower levels of TNF and $PGE_2$ than those from conventional rats after LPS-treatment.

These indicate that the insusceptibility of TNF response in serum of germfree rats *in vivo* is responsible for the reduced response of macrophages *in vitro* to LPS stimulation. High levels of serum corticosterone may be partly responsible for the lower responsiveness of the inflammatory cytokines to LPS in germfree rats.

## Bacterial heat shock protein and mucosal inflammation of gastrointestinal tract

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Association of bacterial heat shock protein 60 (HSP60) with mucosal inflammation of gastrointestinal tract was examined. In flow cytometric analysis, expression of HSP60 on the surface of Helicobacter pylori was correlated with adhesion activity of H. pylori to human gastric epithelial (MKN45) cells. Monoclonal antibody (H20) to H. pylori HSP60 inhibited the adhesion of H. pylori to MKN45 cells. In addition, purufied HSP60 of H. pylori stimulated MKN45 cells to secrete interleukin-8 inducing infiltration of neutrophil. The experiment to examine the effect of HSP60 on gastric mucosa of germ-free (GF) mouse is in progress. Purified HSP60 of Yersinia nterocolitica was injected intraperitoneally into SPF B10A mouse (200ng/mouse, once a week, 8 times). At 2 days after the last administration, the mice were sacrificed for pathological examination. Sever inflammation with infiltration of neutrophil and histological ulceration were detected in the large intestine of the B10A mice. Similar results were obtained when fusion protein of Y. enterocolitica HSP60 and beta-galactosidase was injected. Interestingly, these severe inflammatory changes were not induced in GF mice. These results suggest that bacterial HSP60 might play an important role for induction of chronic inflammation following bacterial infection in the mucosa of gastrointestinal tract.

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Purified HSP60 of *Yersinia enterocolitica* was injected intraperitoneally into SPF B10A mouse (200 ng/mouse, once a week, 8 times). At 2 days after the last administration, the mice were sacrified for pathological examination. Severe inflammation with infiltration of neutrophil and histological ulceration were detected in the large intestine of the B10A mice. Similar results were obtained when fusion protein of *Y. enterocolitica* HSP60 and beta-galactosidase was injected. Intrestingly, these severe inflammatory changes were not induced in GF mice.

These results suggest that bacterial HSP60 might play an important role for induction of chronic inflammation following bacterial infection in the mucosa of gastrointestinal tract.

## Butyrate inhibits and *Escherichia coli*-derived mitogen(s) stimulate DNA synthesis in human hepatocytes

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Bacterial constituents and products of the bacterial metabolism pass from the gut lumen to the portal vein and may influence the homeostasis of the liver. Our aim was to examine whether DNA synthesis of human hepatocyte cell lines is affected by constituents of *Escherichia coli* species or by intracolonic products of bacterial fermentation that reach the liver via the portal vein.

Methods: Supernatant solutions and bacterial cell fractions of *E. coli* K12 and of *E. coli* species from rat fecal flora were separated by multistep centrifugation, French press and microfiltration. The fractions were incubated with a human hepatoma cell line (Hep-G2) and with non-malignant human liver cells (Chang cells). The cells were labelled with thymidine and DNA synthesis was estimated by the labeling index (LI%). DNA synthesis was also estimated following incubation with short chain fatty acids, acetaldehyde and ammonium chloride.

Results: The fractions of *E. coli* from rat fecal flora containing cytosol and nonsoluble intracellular components significantly increased the labeling index in both Hep-G2 and Chang cells. In addition, the supernatant solution significantly increased the LI in Chang cells. Butyric acid reduced DNA synthesis at 10-4 M.

Conclusions: *E. coli* contain mitogenic factors to human hepatocytes in the supernatant solution, cytosol and in nonsoluble intracellular components. Butyrate inhibit DNA synthesis. Thus, soluble bacterial or bacterial-derived mitogen(s) from the intestinal lumen that reach the liver via the portal vein may influence the cell kinetic steady-state of human hepatic cells.

## Effect of the age and germ-free condition on enzyme activities and carbohydrate structures of brush border membranes in rats

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We compared activities of disaccharidases and carbohydrate structures under conventional (CV) and germ-free (GF) conditions in isolated brush borders from small intestines of 18- and 21-day-old and 2-, 6-, and 12-month-old rats. With increasing age lactase decreased more progressively in CV rats than in GF rats. In 2-month-old GF rats the lactase was three times higher than in CV rats. The sucrase and glucomylase increased more rapidly until day 21 in CV than in GF rats. In adult rats the levels of sucrase were higher in GF than in CV rats. ELISA-lectin quantified the binding of *Sambucus nigra* (SNA), *Maackia amurensis* (MAA) and *Ulex europaeus* (UEA I) to NeuNAc(2,6Gal, NeuNAc(2,3Gal and L-Fuc(1,2Gal of glycoconjugates, respectively. SNA and MAA binding showed progressive developmental decrease of sialylation after the age of 18 days. UEA I showed postnatal increase of fucosylation, being maximal at 18 days. Both fucosylation and a 2,3-sialylation was higher in 18-day-old GF than in CV rats. Supported by grants GACR 306/99/1383, 303/99/0197 and 4150-3 from the Min. of Public Health of the Czech Rep.

## The yeast polysaccharide b-glucan induces T cell mediated arthritis in rats

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Identification of molecules which trigger immune mediated inflammatory joint diseases may give clues to disease mechanisms in rheumatoid arthritis (RA). Here we demonstrate that polyarthritis with clinical similarities to RA can be induced in inbred DA rats by a single subcutaneous injection of > yeast b-glucan. Affected joints were infiltrated with T lymphocytes, and a pathogenic role for these cells was clear since the inflammation was completely resolved by depletion of T cells with a monoclonal antibody specific for the ab T cell receptor. Development of T cell mediated arthritis was genetically determined since inbred F344 rats were arthritis-resistant, and genetic influences from several disease-susceptibility loci could be demonstrated in a F2(DAxF344) intercross genotyped for selected genomic markers. Environmental influences appeared also to be important since the susceptibility of gnotobiotic rats decreased after contamination with a yeast microflora. Glucans are abundant environmental factors, but a systematic investigation of different types of glucans revealed that only the b-glucan from yeast was highly arthritogenic. Interestingly, yeast cell walls (zymosan) containing other macromolecules were less pathogenic and whole yeast lysate was almost ineffective for arthritis induction. The host response to b-glucan was investigated, and in situ hybridisation for cytokine mRNA in draining lymph node cells revealed expression for TNF-a and IFN-g but not IL-4. Furthermore, ELISA analysis of supernatants from whole blood stimulated 24h in vitro with b-glucan demonstrated that the TNF-a protein is rapidly produced in response to the arthritogen. We speculate that this early TNF-a production is evidence for an initial response of the innate immune system which subsequently also involves adaptive lymphocytes and renders some T cells arthritogenic. Further dissection of glucan-induced arthritis may unravel the intriguing mechanisms whereby a microbial adjuvant molecule can trigger a model disease for RA.

## Obtention of infective larvae of *Strongyloides venezuelensis* under axenic conditions

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To study a possible influence of the intestinal microbiota on the course of strongyloidiasis in mice, a method for the obtention of axenic infective larvae of *Strongyloides venezuelensis* was developed and the resulting parasites were inoculated in germfree and conventional mice. The larvae were recuperated from a coproculture performed at 27°C during 3 days using feces from AKR/J mice infected with *S. venezuelensis*. All the tested disinfections were carried out in two steps. First the L3 larvae were submitted to sodium hypochlorite 0.25% during 10 min. Then, the parasites were thoroughly washed with sterile distilled water and submitted during 30 or 60 min to a second disinfection step using various antibiotic combinations diluted in distilled water. Axenic status and viability were determined, respectively, by cultures in thioglycolatte medium and brain heart infusion broth and by subcutaneous inoculation of a 0.5 ml suspension containing 500 L3 larvae in germfree and conventional NIH mice and conventional AKR/J mice. Axenic conditions and viability were obtained only when, after the first disinfection with sodium hypochlorite, L3 larvae were submitted during 30 min to a solution containing penicillin (300 U/ml) and ceftazidime (1 mg/ml).

## Lincosamide antibiotics enhance murine neutrophil microbicidal activity

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Lincosamide antibiotics, lincomycin and clindamycin, have been developed by Pharmacia & Upjohn and are used to treat bacterial infections, including those caused by anaerobes. Both of these antibiotics are known to be concentrated by host phagocytic cells, and we have previously reported that they enhance macrophage fungicidal activity, *in vivo* clearance of yeasts and murine contact sensitivity to dinitro-fluorobenzene (JAC 23:721-728, 1989). Currently we have studied lincosamide drug effects on murine peritoneal-derived neutrophil microbicidal activity for eukaryotic candidal yeasts. Both drugs boost neutrophil antifungal activity with lincomycin having the greater effect. Lincosamide effects on yeast uptake by neutrophils and optimal target:effector (yeast:neutrophil) ratios for fungicidal activity have been examined. These findings demonstrate that while use of antibiotics may

reduce a hosts colonization resistance through effects on the normal flora, there are also positive antibiotic-mediated effects on the immune system that are beneficial to the host.

This work was supported in part by the Max Baer Heart Fund, Fraternal Order of Eagles.

## Anti-carcinogenic effect of isoflavones and their metabolism by intestinal flora in mice with MMTV-induced breast cancer

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For the purpose of investigating the anti-carcinogenic effect of isoflavones against breast cancer, biochanin A and didzein, typical isoflavones, were given to both germfree and conventional GR mice with breast cancer induced by a mouse mammary tumor virus (MMTV). Three kinds of a diet, diets containing 200mg/kg of daidzein or biochanin A, and CA-1 as a control, were prepared. The animals were divided into six groups as follows; 1) conventional-biochanin A diet (Cv-b), 2) conventional-daidzein diet (Cv-d), 3) conventional-control diet (Cv-c), 4) germfree-biochanin A diet (Gf-b), 5) germfree-daidzein diet (Gf-d), 6) germfree-control diet (Gf-c). Urine biochanin A and daidzein level were measured. Urine genistein and equol levels, which were metabolized by intestinal flora, were also measured. The animals were observed up to 15 months old and the cumulative incidence of breast cancer was determined. Urine biochanin A and genistein level in Cv-b was higher than in Cv-c. Urine daidzein and equol level in Cv-d was higher than in Cv-c. Only urine biochanin A level in Gf-b was higher than in Gf-c, and only urine daidzein level in Gf-d was higher than that in Gf-c. The cumulative incidence of breast cancer was 35.2% in Cv-b, 81.5% in Cv-d, 84.6% in Cv-c, 67.1% in Gf-b, 34.0% in Gf-d and 23.5% in Gf-c. It is conceivable that biochanin A may exhibit an anti-carcinogenic effect against breast cancer as a precursor of genistein, and that genistein may be a potential anti-carcinogenic substance against breast cancer.

## Human intestinal bacteria and bile acid transformation in gnotobiotic mice

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Bile acids are transformed by intestinal microflora. Secondary bile acids including deoxycholic acid and lithocholic acid, the deconjugated and 7$B&A(J-dehydroxylated forms of conjugated primary bile acids, appear to show a strong correlation with colorectal cancer. However, the role of intestinal bacteria in the transformation of bile acids in vivo is not yet clear. We produced ex-germfree (ex-GF) mice and gnotobiotic (GB) mice associated with human intestinal bacteria or various kinds of human fecal suspension to clarify which bacterial groups are responsible for bile acid transformation in vivo. GF mice were orally inoculated with human fecal dilution or various components of human feces. In the cecal contents of ex-GF mice associated with human fecal dilutions of 10-2 and 10-6, or the anaerobic growth from a dilution of 10-6, free-form bile acids accounted for more than 80% of total bile acids and deoxycholic acid for about 20% of total bile acid. However, when GF mice were associated only with clostridia, free-form bile acids made up less than 40% of total bile acids, but the percentage of secondary bile acids was the same as in the other groups. These results indicate that clostridia in human feces may play an important role in 7$B&A(J-dehydroxylation of free-form primary bile acids in the intestine.Other results have suggested that bacteroides is the main bacterial group for deconjugation of bile acids.

## 40–100 kD protein(s) of *Helicobacter pylori* stimulate DNA synthesis in epithelial cell lines without affecting apoptosis

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Previous studies show that water extracts of *Helicobacter pylori* increase DNA synthesis in a small intestinal epithelial cell line. Our aim was to try to identify mitogenic factor(s) in a water extract of a *Helicobacter pylori* strain and to examine their ability to induce apoptosis *in vitro*.

Methods: IEC-6 and FHs74 cells were incubated with a water extract of *H. pylori* (cytotoxic strain 88-23) or with 6 protein fractions obtained by gel filtration. The cells were labeled with tritiated thymidine and DNA synthesis was evaluated by the labelling index (LI%). The proportion of IEC-6 cells in apoptosis and/or necrosis was evaluated by flow cytometry in cells exposed to fluorescein isothiocyanate (FITC)-labelled annexin-V and propidium iodide. The caspase activity was also determined to detect early apoptotic events.

Results: The water extract of *H. pylori* increased DNA synthesis in both epithelial cell lines. A marked stimulation of DNA synthesis was observed in IEC-6 cells incubated with fraction II-containing proteins of a molecular weight ranging between 40–100 kD. Neither the water extract of *H. pylori* 88-23 nor the protein fraction II (40–100kD) induced apoptosis in IEC-6 cells.

Conclusion: A water extract of *H. pylori* 88-23 and a protein fraction containing proteins with a molecular weight of 40–100 kD stimulate DNA synthesis in small intestinal cell lines. Apoptosis was unaffected which indicate that the *H. pylori*-derived mitogen(s) have the capacity to directly enhance epithelial cell proliferation.

## Gene(s) from the cag pathogenicity island of *Helicobacter pylori* are responsible for the stimulation of DNA synthesis in epithelial cell lines

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Background: Studies *in vitro* show that water extracts of *Helicobacter pylori* increase DNA synthesis in epithelial cells. Our aim was to investigate whether these actions are related to the cag pathogenicity island (PAI).

Methods: IEC-6 and FHs 74 cells were incubated for 24 h with different dilutions of a water extract of strains *H. pylori* lacking cag PAI (A), carrying cag PAI (B) and a strain containing parts of the cag PAI (C). All the strains were collected from the same patient affected by gastric carcinoma. The cells were labeled with tritiated thymidine and processed for autoradiography. DNA synthesis was evaluated by the labeling index (LI%). The proportion of IEC-6 cells undergoing apoptosis and/or necrosis was evaluated by flow cytometry using fluorescein isothiocyanate (FITC)-labeled annexin-V and propidium iodide. *In vitro* caspase activity was also determined as an alternative method for detection of apoptosis.

Results: The water extract of *H. Pylori* strain B markedly increased DNA synthesis in both epithelial cell lines. A marked stimulation of DNA synthesis was also observed in FHs-74 cells incubated with strain C containing parts of the cag PAI. Interestingly, there was no stimulation of DNA synthesis in IEC-6 cells or FHs-74 incubated with the strain A, lacking cag PAI. None of the water extracts of H. pylori induced apoptosis in IEC-6 cells.

Conclusion: The ability of *H. pylori* to stimulate epithelial cell proliferation is genetically regulated. The gene(s) responsible for the mitogenic property of *H. pylori* is/are located in the cag pathogenicity island. The water extracts of *H. pylori* did not affect apoptosis indicating the microorganism contains mitogen(s) that directly enhance epithelial cell proliferation *in vitro*.

## The effect of administration of Lactobacillus reuteri on the establishmental pattern of normal intestinal functions in rats

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The last decades have much intrest been focused on the use of ''live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance''. The aim of this investigation was to evaluate if inoculation with Lactobacillus reuteri before being inoculated with a normal environmental flora, would improve the establishmental pattern of some MACs (Microflora Associated Characteristics) in rats. Germfree rats were mono-inoculated with L. reuteri for ten days prior to inoculation by random with the flora occuring in a conventional animal room or by direct daily contact with conventional visitor rats. Fecal samples from the animals were collected during four weeks after being brought out from the isolator and investigated with regard for the following MACs; conversion of cholesterol to coprostanol and bilirubin to urobilin, degradation of tryptic activity, mucin and β-aspartylglucin and the short-chain fatty acid pattern. It was found that the establishmental patterns of the MACs investigated were more smoth, uniform and consistent in those rats preinoculated with so called probiotic microbes as compared with not primed animals.

## Bacterial wall components and heat-shock protein 60 stimulate cell proliferation in intestinal epithelial cells

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Previous studies indicate that the microflora contains mitogens to intestinal epithelial cells. Our aim is to examine whether cell wall components of Gram-negative and Gram-positive bacteria and heat-shock protein-60 (hps-60) of *Escherichia coli* influence cell proliferation in intestinal epithelial cells. LS-123 and IEC-6 cells were incubated with: (a) lipothecoid acid (LTA) from *Streptococcus faecalis*; (b) peptidoglycan from *Staphyloccocus aureus*; (c) lipopolysaccharides (LPS) from *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomona aeruginosa*; (d) lipid A from *Escherichia coli*; (e) HSP-60 from *Escherichia coli*. The cells were labeled with thymidine and DNA synthesis was estimated by the labeling index. The influence of HSP-60 on apoptosis was also evaluated. In an additional study, human explants from normal colonic mucosa were incubated with HSP-60 from *Escherichia coli*. Metaphases were arrested with vincristine and the number of mitoses per crypt was determined. Cell wall components of Gram-positive bacteria (LTA from *Streptococcus faecalis* and peptidoglycan from *Staphylococcus aureus* moderately increased the DNA synthesis in IEC-6 and LS-123 cells. Cell wall components of Gram-negative bacteria (LPS of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomona aeruginosa* and lipid A of *Escherichia coli*) increased the labeling index in both cell lines. HSP-60 of *Escherichia coli* markedly increased DNA synthesis in the intestinal cell lines and it increased the number of arrested mitoses in the human colonic crypts. HSP-60 did not affect apoptosis. This study identifies bacterial mitogens to rat and human intestinal epithelial cells, which further support a role for the microflora in the regulation of cell proliferation in health and disease.

## Antitumorigenecity of chloroform-resistant bacteria derived from intestinal microflora of conventional mice

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The development of spontaneous polyposis in the small intestine of BALB/c germfree (GF), conventionalized (CVz), and gnotobiotic (GB) mice which were associated with chloroform-resistant bacteria (CRB) and fusiform bacteria (FB) was studied. CRB and FB were inoculated orally to GF BALB/c female mice 4 (CRB-Y-GB, FB-Y-GB) and 30 (CRB-A-GB, FB-A-GB) weeks of age, respectively. The CRB was prepared by treating fresh cecal content of conventional BALB/c mice with chloroform. FB were isolated anaerobically from CRB. When the animals were 12 months old, they were autopsied carefully for the number and the size of polyps in the small intestine. The incidence of polyposis and the number of polyps per mouse were significantly lower for CVz (36%, 0.7), CRB-Y-GB (18%, 0.2), FB-Y-GB (45%, 0.6), CRB-A-GB (26%, 0.3) and FB-A-GB (53%, 0.8) mice than for GF mice (76%, 2.7). The mean sizes of polyps, which varied from 1.0 to 1.3 mm, were not significantly different between the GF group and other groups. The present result showed that the polyposis was suppressed by the presence of the CRB or FB in GB BALB/c mice. The suppression of polyposis was effective in CRB-A-GB mice even when the CRB were inoculated at 30 weeks of age. This suggested that antitumorigenecity of CRB was not only preventive but also curative in the polyposis in the small intestine of BALB/c mice. Farther study is needed to manifest the mechanism of inhibitory effect of CRB on spontaneous multiple polyps in the small intestine of this unique animal model.

## Platelet interactions with candidal fungi

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Megakaryocytes produce cell fragments called platelets which circulate in the bloodstream and which respond to tissue injury and inflammatory processes. Platelets are known to contribute to clotting and wound healing and to play a role in host response to

infectious agents. However platelet–fungus interactions have not been well studied. Recently Tewari and associates have reported that *Histoplasma capsulatum* yeast phase cells are ingested and killed by human platelets. We have studied the interaction of human platelets from four individuals with the pathogenic yeasts *Candida glabrata*, *Candida tropicalis* and *Candida lusitaniae*. We will report our results from experiments examining various target:effector (yeast:platelet) ratios and incubation times. It appears that platelets play an important role in helping to control fungus infections involving the host blood vascular system.

This work was supported in part by the Max Baer Heart Fund, Fraternal Order of Eagles.

## *Saccharomyces boulardii* stimulates the host phagocytic system and type 1 cytokine production

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*Saccharomyces boulardii* is a non-pathogenic yeast that has been prescribed on an empirical basis in Western Europe based on its anti-diarrheal properties. One of the mechanisms suggested to explain the beneficial effects of the ingestion of living microorganisms (probiotics) is stimulation of immune responses. The influence of *Saccharomyces boulardii* on the host was tested by assessing: (i) liver histological examination, (ii) activity of host phagocytic system by measuring *Escherichia coli* $B_{41}$ clearance from the blood and (iii) serum type 1 cytokine levels after the bacterial challenge. The effect of the yeast was evaluated comparing germfree Swiss/NIH mice monoassociated with the probiotic for 10 days with germfree control groups. The number of Küppfer cells was significantly higher in monoassociated mice than in germfree controls after 10 days of monoassociation. In *S. boulardii*-monoassociated mice the clearance of *E. coli* $B_{41}$ injected in the tail vein was higher when compared to germfree controls. It was observed an augmentation in the number of Küppfer cells only in the livers of germfree mice after the bacterial challenge. However, TNF-$\alpha$ and IL-12 serum levels were higher at earlier time points in monoassociated mice when compared to germfree mice. Consequently, IFN-$\gamma$ serum levels at 4 and 8 hours after *E. coli* $B_{41}$ challenge were significantly higher in *S. boulardii*-monoassociated mice. These results show that the yeast *S. boulardii* increases the number and function of phagocytic cells of the host monoassociated with this probiotic which can be of interest to improve the resistance to enteropathogenic bacterial infections.

## Anti secretory protein and feed induced lectines in sow and suckling piglets

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Anti Secretory Protein (ASP), produced and secreted from the pituitary gland in response to enterotoxin activity inhibits hypersecretion of water and electrolytes in the small intestine. There is a big intra sow variation in sow milk ASP-concentration. Low levels of ASP in sow milk were correlated with high occurrence of neonatal diarrhoea in the litter. In contrast to the immunoglobulines, ASP is transferred to the fetuses via the placenta. The active portion of ASP is not digested by the piglets after gut closure, but is transferred to the blood of the piglet. The production and release of a similar protein, Feed Induced Lectines (FIL), can be induced by a proper mix of sugars, sugar alcohols and pure amino acids in the sow diet. FIL has the same anti secretory effect in the intestine as ASP. The FIL concentration in the blood of the piglets is negatively related to the frequency of postweaning diarrhoea, i.e. high levels correspond to a low diarrhoea incidence, while a low FIL-level corresponds to a high diarrhoea incidence. At weaning, however, FIL received from the sow milk decreases very rapidly. Thus, a high FIL status of the piglet during the critical phase of weaning can be secured by the weaning diet composition.

## Antagonism against *Vibrio cholerae* through the production of diffusible substance by bacterial components in the human fecal microbiota

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The potent resistance of gastrointestinal ecosystem of conventional rodents to colonization with *Vibrio cholerae* is well known. On the contrary, the resident digestive microbiota of the major part of human is not so efficient. However, the cholera vibrios sometimes survive, probably in low silent populations, in the small intestine of chronic carriers or pass through the gastrointestinal tract of few individuals without causing diarrhea or colonization. To understand the two last situations we studied on plates (ex vivo test) the frequency of appearance of an inhibitory halo against *V. cholerae* from feces of 92 healthy volunteers (40 females, 52 males), 4–61 years old. The frequency of inhibitory halo was 20.6% in the whole group. An apparently ($P = 0.079$) higher percentage was observed in the age range from 20 to 40 years (27.3%) when compared with range from 4 to 19 years (10.7%) but not ($P = 0.385$) with range from 41 to 61 years (20.0%). Frequency was significantly higher ($P = 0.005$) in males (30.8%) when compared with females (7.5%). The dominant microbiota of a volunteer whose feces produced an inhibitory halo was isolated by decimal dilution in an anaerobic chamber. Twenty-six apparently different morphologies were associated with germfree NIH mice. One week later, the inhibitory test showed an antagonistic halo around the feces of the associated animals but not for the axenic ones. Antagonism against cholera vibrio through diffusible compounds produced by bacterial components of the fecal microbiota was found in part of the human population.

## Homo- and autobiotics prepared with using selective decontamination of biospecimen

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It is universally recognized that enhancing the "friendly" bacteria can promote long-term host health. Probiotics and functional food products (FFPs) containing living bacteria with proved ability to increase colonization resistance, to produce hypocholesterolemic, antiallergic, antyhypertensive, anticarcinogenic and other positive effects have already introduced into clinic practice. The main defect of such probiotics and FFPs is that they work out without consideration of immonologic intolerance and colonization resistance of future host with living microorganisms introduced orally. So we recommend to construct probiotics and FFPs on the base of bacteria possessing species and/or individual compatibility with future recipient and its colon microorganisms. For this the feces (the source of "friendly" bacteria) are treated with immunooglobulins received from blood of certain species of

animals, birds or humans (for making of homobiotics) or blood serum of definite human being (for construction of autobiotics). Thanks to such selective decontamination and using of special selective lactic acid bacteria media followed it's possible to eliminate all undesirable bacteria and to make probiotics and FFPs containing living bacteria compatible with future recipient. The example will be given of using of that mean for working out of the homobiotics for settling of newborn chickens and autobiotic for adult human being.

## Effect of milk gangliosides on the survival of gnotobiotic mice infected with enterohemorrhagic E. coli

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In 1996, there were several large outbreaks of enterohemorrhagic E. coli (EHEC) O157:H7 in Japan. More than 9,000 people were infected, and 11 of the infections were fatal. Verotoxins produced by EHEC were responsible for the deaths due primarily to hemolytic uremic syndrome. On the other hand, glycolipids containing sialic acid are called gangliosides (GA), and a specific GA on intestinal mucosal cells is known to mediate the action of cholera toxin. The aim of the present study was to examine the effect of milk GA on the pathogenicity of EHEC in germfree (GF) mice.

Young adult male and female BALB/c, GF mice were used. Mice were given sterilized GA with tap water ad libitum. EHEC ($8.5 \times 106$ cfu/mouse) was administered per os using a stomach probe on day 3 after the mice were placed on the GA solution. The effects of GA on the survival rate and EHEC adhesion to intestinal mucosal surfaces in EHEC-infected GF mice were examined. Survival rates of EHEC-infected GF mice were 30% in the absence of GA, and 70% when administered GA. However, there was no significant difference between the number of adherent bacteria in the GF mice whether given or not given GA. This result indicated that GA is not capable of functioning as inhibitors of EHEC adhesion to intestinal mucosal surfaces. In order to explain the difference in survival rates, the effects of EHEC-produced verotoxin are discussed.

## The influence of cecal microflora on the digestion of ganlgioside GM1

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Sphingolipids modulate cell growth, differentiation, and transformation. Ganglioside GM1, one of the sphingolipids, inhibits 1,2-dimethylhydrazineinduced colon cancer in CF1 mice. However, little is nownm about the disgestion and uptake of ganlgioside GM1. The present study describes the influence of cecal microflora on ganglioside GM1. In experiment 1, 0.5 ml GM1 solution (4mg/ml), or physiological saline solution as control, was orally administered to each of the four ICR mice of each group. After 16 hours, the ceca were excised and the cecal contents were extracted with chloroform–methanol–water (4:8:3). The extracts were analyzed with HPTLC and ganglioside GM1 was not detected in the GM1 or control groups. In experiment 2, the cecal content from the ceca of ICR mice were aseptically transferred to test tubes, and 0.5 ml of ganglioside solution (1mg GM1/ml) was mixed with 0.5 ml cecal solution. The tubes were anaerobically incubated for 16 hours at 37°C. The raction mixtures were extracted with chloroform–methanol–water (4:8:3), and the extracrts were used for HPLC and HPTLC analysis. The rate of hydrolysis of ganglioside GM1 by cecal microflora was about 71.5%. The major metabolite was ganglioside GM2. Ganglioside GM2, hydrolyzed from ganglioside GM1, in the cecum, might related with the reduction in 1,2-dimethylhydrazine-induced premalignant lesions.

## Concurrent Candida albicans and murine cytomegalovirus infection in germfree mice

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An increased frequency of bacterial or fungal infections occurs in transplant recipients with primary cytomegalovirus (CMV) infection. To examine this phenomenon, a number of studies have been performed employing murine CMV (MCMV). The present study was designed to examine the effect of concurrent Candida albicans (CA) and MCMV infection.

The mice were injected intraperitoneally (ip) with a sublethal dose ($5 \times 105$ pfu/mouse) of MCMV, followed by CA given ip at a dose of $5 \times 107$ cfu/mouse after one day. Survival rates of the MCMV and CA infected mice were 0% in GF mice and 65% in specific pathogen-free (SPF) mice. In GF mice infected with both MCMV and CA, the MCMV titer in various organs were similar to those in GF mice infected with MCMV alone. The viable counts of CA in organs remained remarkably high until death in GF mice infected with both MCMV and CA, suggesting the cause of death to be severe generalized infection by CA rather than infection by MCMV. In order to explain these findings, we have set out to examine the number of neutrophils in heart blood of super-infected mice. In GF mice, neutrophil counts are more markedly depressed than in SPF mice, and the recovery from the depression is slow. Thus it was believed this slow increase of neutrophils was one factor contributing to the high mortality of GF mice with concurrent infection.

## The translocating bacteria in rats with acute liver injury, and the probiotic effect of *Lactobacillus plantarum* DSM 9843

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Bacterial translocation was measured in rats with acute liver injury. The rats were divided into two groups, and before initiation of liver injury by intra-peritoneal injection of D-galactosamine, one group was rectally administrated *Lactobacillus plantarum* DSM 9843 daily for 8 d and the other saline for the same time. Samples were collected, 24 h after initiation of liver injury, for viable count of portal and arterial blood, mesenteric lymph nodes, liver tissue, and colonic and cecal mucosa. Randomly selected colonies from the countable plates were isolated and typed by Randomly Amplified Polymorphic DNA (RAPD), and one isolate of each RAPD-type was selected for 16 S rRNA gene sequencing. Administration of *Lb. plantarum* DSM 9843 significantly lowered the incidence of translocation, and decreased the viable counts from extra-intestinal sites.

## Lactobacillus reuteri and intestinal integrity

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Rodent models have helped to demonstrate that Lactobacillus reuteri plays a central role in maintenance of intestinal integrity. For example colitis rat models (induced by acetic acid or methotrexate) and acute liver failure have been used to shown that L. reuteri improves the permeability, decreases bacterial translocation and intestinal pathogens in the gastrointestinal (GI) tract. Administration of L. reuteri to immuno-deficient mice decreases Cryptosporidium parvum infection.

The information in this presentation deals with L. reuteri host protection from two pathogens: Salmonella typhimurium or Cryptosporidium parvum. L. reuteri BALB/c mice were challenged with S. typhimurium, L. reuteri improved significantly livability and decreased S. typhimurium translocation and gut epithelium damage. In an inflammatory bowel disease (IBD) model using TCR alpha deficient gnotobiotic mice, L. reuteri significantly decreased cecal inflammation scores at four and seven weeks post crypto challenge. Significantly lower crypto infection was also observed in the cecum and ileum at four and seven weeks, respectively.

These results support the role of L. reuteri in maintaining intestinal integrity is rodents such effect has been previously observed in humans and avians and non rodent mammals.

## Global study of the composition of the intestinal bacterial flora in healthy volunteers

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The human intestinal tract contains a complex ecosystem consisting mainly of obligatory anaerobic bacteria, usually referred to as the *normal* flora. The composition of this flora plays an important role in human health and disease. We have developed a number of fluorescent 16S rRNA targeted oligonucleotide probes for the numerically most important groups of bacteria in the human intestine. A suitable dilution of a faecal sample is treated with paraformaldehyde and applied to a $1 \times 1$ cm well on a gelatin-coated microscopic slide. After fixation with 96% ethanol, slides can be stored at room temperature until hybridization with 5–7 specific probes that account for approximately 80% of the flora. To facilitate detection and enumeration, we have combined whole cell fluorescent in situ hybridization (FISH) with automated microscopic image analysis and processing. In each sample twenty-five fields of view each containing 50–200 objects are measure in 15 min. This methodology is particularly suitable to determine and to compare the bacterial composition in the intestinal tract of human volunteers in different countries.

Call for collaboration: Interested groups will be provided with a kit containing gelatin-coated slides, paraformaldehyde and an extensive protocol for application of faecal samples of 18 volunteers to the slides. Slides should then be sent to the Groningen laboratory for hybridization and subsequent image analysis and processing. In this first study we will compare the bacterial composition of healthy volunteers aged 18–55 years.

## Macrolide antibiotic effects on murine neutrophil function

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Neutrophils participate in non-specific responses to infectious agents known as inflammation. Many antibacterial antibiotics have been reported to boost immune responses, especially chemotaxis and microbicidal activity as related to neutrophil function. Macrolide antibiotics studied including erythromycin (Sigma), azithromycin (Pfizer), troleandomycin (Pfizer) and clarithromycin (Abbott) were of interest as they are known to be intracellularly concentrated by neutrophils. Since macrolide antibiotics inhibit prokaryotic protein synthesis, we used eukaryotic *Candida* yeast targets and peritoneal-derived neutrophils from AKR/J mice to study drug-induced immune effects. As a group the macrolides enhanced neutrophil killing of yeast targets, although results found with individual drugs varied. These findings compare favorably with our previous studies showing antibiotic enhanced macrophage fungicidal activity. These findings also show that antibiotic-mediated effects on immune cells can benefit the host and perhaps offset the decrease in colonization resistance caused by antibiotic induced alterations of the host normal flora.

This work was supported in part by the Max Baer Heart Fund, Fraternal Order of Eagles.