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INFLUENCE OF THE MICROFLORA ON GASTROINTESTINAL NITRIC OXIDE GENERATION

STUDIES IN NEWBORN INFANTS AND GERM-FREE ANIMALS

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To my son

"- Do you know where the idea of a labyrinth come from?
- It was the ancients Mesopotamians. They pulled out animal intestines… and used the shape to predict the future. They admired the complex shape of intestines. So the prototype for labyrinths is, in a word, guts. Which means that the principle for the labyrinth is inside you. And it correlates to the labyrinth outside… Things outside you are projections of what's inside you, and what's inside you is a projection of what's outside. So when you step into the labyrinth outside you, at the same time you're stepping into the labyrinth inside. Most definitely a risky business."

Haruku Murakami, Kafka on the shore.

Abstract

Interactions between intestinal bacteria and the host play an important role in physiological regulation of gut function and in development of various diseases. Nitric oxide (NO) exhibits a variety of biological actions in the gut including regulation of regional blood flow, gut motility, water and electrolyte transport and immunity. In patients with inflammatory bowel disease (IBD) the mucosal production of NO from NO synthases is greatly increased but its role in the pathophysiology of IBD is still unclear. Denitrifying bacteria in soil generate NO from nitrate (NO_3^-) and nitrite (NO_2^-) as a part of the nitrogen cycle. In this project we wanted to investigate whether the micro-organisms residing in the gastrointestinal (GI) tract could contribute to NO generation and under which conditions this would occur *in vivo*.

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

We started this project by monitoring the initial bacterial colonization in newborn infants with repeated measurements of intracolonic hydrogen gas (H_2), fecal short-chain fatty acids (SCFA) and NO. These markers were virtually undetectable at birth but increased in a particular pattern - bacterial products (H_2 and SCFA) appeared first followed by NO some days later. Interestingly, in some apparently healthy infants colonic NO levels increased to levels similar to those seen in adults with inflammatory bowel disease, indicating a vivid activation of the immune system in response to the emerging bacterial flora.

Next we investigated if bacteria could be an alternative source of gastrointestinal NO in addition to the mucosa. We found that in conventional rats, NO levels were distinctly compartmentalized with very high levels in the stomach, intermediate levels in the cecum and lower levels in the small intestine and colon. In contrast, in germ-free rats, NO was low throughout the gastrointestinal tract. When we fed rats nitrate, gastric NO increased greatly in conventional but not in germ-free animals, thereby confirming nitrate to be a substrate for bacterial NO generation. We went on to demonstrate that lactic acid producing bacteria (Lactobacilli and Bifidobacteria) can generate considerable amounts of NO from nitrite *in vitro*. A combined mixed faecal flora was capable of NO generation not only from nitrite but also from nitrate.

In the final study we demonstrate that intestinal NO generation can be stimulated *in vivo* by dietary supplementation with substrate (nitrate) and lactobacilli. Furthermore, *in vitro* studies show that the generation of NO by some probiotic bacteria can be counteracted by rapid NO consumption by other strains (*E. coli* and *S. aureus*).

We conclude that commensal bacteria can be a significant source of NO in the gut in addition to the NO produced in the mucosa. NO generation by gut bacteria differ profoundly from classical mammalian synthesis via NO synthases as bacteria use nitrate and nitrite as substrates instead of L-arginine. Future studies will clarify the biological role of the bacteria-derived intestinal NO in health and disease and if an imbalance in generation *vs* consumption has any significance in the patho-physiology of intestinal disorders. Direct minimally-invasive measurements of intestinal gases including NO and H_2 may also be useful to study the dynamics of the microbial colonization process and host-microbial interactions early in life.

Keywords: Nitrite, nitrate, IBD, lactobacilli, probiotics, germ-free, SCFA, H_2, *newborn infants*

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I T Sobko, M Norman, E Norin, LE Gustafsson, JO Lundberg.
Birth-related increase in intracolonic hydrogen gas and nitric oxide as indicator of host-microbial interactions.
Allergy 2005 Mar; 60(3): 396-400

II T Sobko, C Reinders, E Norin, T Midtvedt, LE Gustafsson, JO Lundberg.
Gastrointestinal nitric oxide generation in germ-free and conventional rats.
American Journal of Physiology - Gastrointestinal and liver Physiology. 2004 Nov; 287(5): G993-7

III T Sobko, C Reinders, E Å Jansson, E Norin, T Midtvedt, JO Lundberg.
Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite.
Nitric Oxide: Biology and Chemistry. 2005 Dec; 13(4): 272-8

IV T Sobko, L Huang, T Midtvedt, L Gustafsson, E Norin, M Norman, E Å Jansson and JO Lundberg
Generation of NO by probiotic bacteria in the gastrointestinal tract.
Manuscript (Submitted)

Table of contents

ABBREVIATIONS

cNOS	constitutive nitric oxide synthase
CFU	colony-forming unit
Conv	conventional
eNOS	endothelial nitric oxide synthase
GF	germ-free
GI	gastrointestinal
H_2	hydrogen gas
H_2O_2	hydrogen peroxide
IBD	inflammatory bowel disease
iNOS	inducible nitric oxide synthase
LAB	lactic acid producing bacteria
LGG	lactobacillus rhamnosus
L-NAME	N^G-nitro-L-arginine methylester
L-NMMA	N^G-monomethyl-L-arginine
N_2	dinitrogen
NEC	necrotizing enterocolitis
NF-κB	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO_2^-	nitrite
NO_2^*	nitrogen dioxide in the excited state
NO_3^-	nitrate
O_2^-	superoxide
$ONOO^-$	peroxynitrite
ppb	parts per billion
SCFA	short chain fatty acid
SNOs	S-nitrosothiols

Introduction

The world we live in is not sterile; bacteria are literally everywhere, they are found in the air, water, soil, plants, animals and in man. Humans are covered with microorganisms on all surfaces and have approximately 10 times more prokaryotic (mainly bacteria) than eukaryotic cells[1]. Through the evolutionary development both parts have learnt to coexist, and do so with mutual benefit. The intestinal flora can elicit a number of effects in the gut, however the mechanisms behind this are not entirely understood[2]. Communication between bacteria and the host occurs via receptors and through the secretion of chemical mediators. The studies presented in this thesis suggest that some common intestinal bacteria can generate nitric oxide (NO), a potent biological messenger with a variety of known physiological functions. The main focus here has been to characterise NO generation by bacteria but we have also allowed ourselves to speculate on its possible physiological relevance.

Bacteria and humans

Each human carries an individual heterogeneous microbial ecosystem containing up to 10^{12} colony-forming units (CFU) of bacteria[3,4]. While some of these bacteria are just passing through, others are permanent residents of the specific gut compartments[5]. Within each compartment they live either close to the epithelial cells or in the lumen [6, 7] (Fig.1).

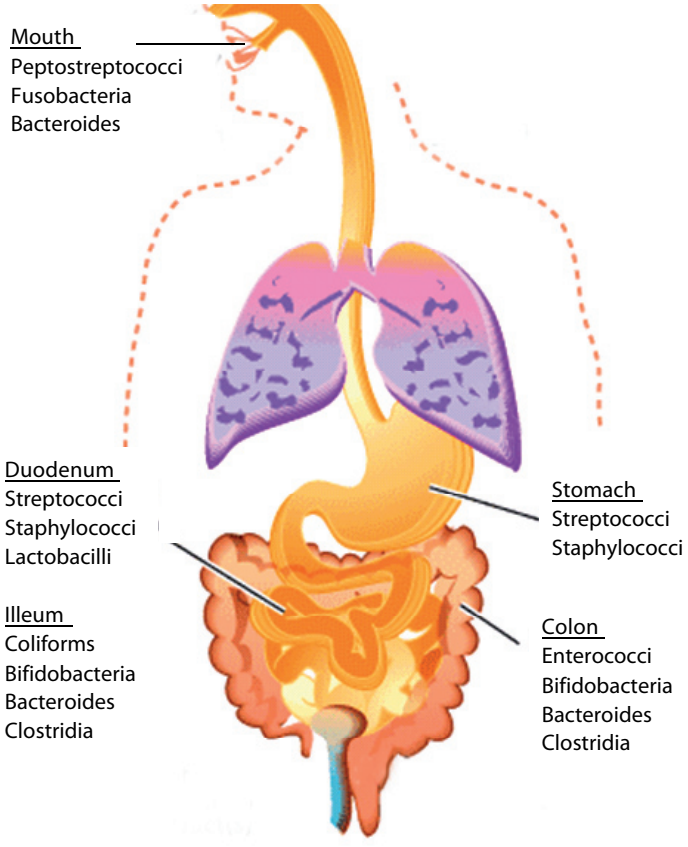

Figure 1. *Overview of bacterial species present in the GI tract, modified.*

The normal flora of the human GI tract starts with the oral cavity's approximately 500 different kinds of bacteria[8]. The mouth is a reservoir, which supplies the rest of the GI tract with a multitude of different microbes. Further down, in the acidic stomach, only a few species can survive, a good example being *Helicobacter pylori*. The small intestine successively becomes a relatively favorable environment for bacteria, although the flow rate is high and bacteria get washed down very quickly, preventing an abundant colonization (10^6 bacteria per ml)[9]. In the colon, due to its slower transit time, the numbers increase up to 10^9-10^{11} bacteria per ml and up to 50% of the colon content in an adult consists of microbes. The environment becomes gradually anaerobic along the gut and up to 99% of the flora in the large intestine is strict anaerobic[10]. The bacterial flora here includes several hundred species, of which Enterobacteria, Eubacteria, Streptococci, Clostridia and Lactobacilli, Bacteroides and lactic acid-producing Bifidobacterium can be mentioned[11].

Newborn infants

After birth the newborn infant meets a highly contaminated extra-uterine environment, which requires a well-functioning immune system to avoid serious systemic and gastrointestinal diseases. However, in order to "switch on" the host immunity, the gut must first be exposed to the colonizing bacteria [12,13].

When entering the world full of bacteria, through the birth canal, a germ-free infant swallows a mouthful of mixture from the mother's vaginal and faecal flora including Lactobacillus, Bifidobacterium, E.coli and Enteroccoccus [4,15]. During their first days of life, a continuous flow of different bacteria join the gut flora - a typical one is Staphylococcus aureus from the mother's nipple and contacts with the surroundings[16]. The population of each species is strictly regulated by competition for nutrients and space. Thus, one group of organisms after another establishes and provides a first line of defense against potential pathogens. This process is dependent on complex regulatory mechanisms, which involves the microbes themselves, the environment, diet, and the innate and adaptive immunity[17,18]. It continues for several years, until the intestinal flora is more or less stabilized/completed with a large variety of microorganisms in particular niches[9]. In the case of Caesarian delivery the infant will obtain a different flora, a "Caesarian baby's bacterial breakfast": including hospital-acquired Clostridia and Streptococci[19].

Substances in breast milk have many effects on the neonatal intestines [20,21]. They also facilitate the establishment of bifidobacteria[22,23], which already after a few weeks make up over 90% of a breastfed neonate's intestinal flora. Bifidobacteria make the gut environment acidic, creating a barrier against infection. Formula-fed infants establish a slightly different intestinal flora initially, and, as a consequence, they are probably more prone to gut infections[24, 25].

Role of bacteria in the GI tract

The intestinal microflora carries out a number of important functions in the GI tract, many of which are known today and others likely to be discovered. A good model to study host-microbial interactions has been by using germ-free animals. Considerable insights into the role of bacteria in the gut have been gained using this unique research environment[26-29].

The normal intestinal flora produces essential nutrients. As an example, bacteria synthesize vitamin K and other vitamins that cannot be synthesized by the host[30], and they generate and metabolize carcinogens[31,32]. Moreover, bacteria are involved in the immediate postnatal development, promoting for example the development of gut capillary networks[33] and modulation of endocrine cells in the gastrointestinal mucosa[34]. In the colon, bacteria are involved in numerous metabolic activities, including fermentation of carbohydrates, utilization of nitrogenous substances, and biotransformation of bile acids and other steroids[28,32]. Short chain fatty acids (SCFAs) are products of intestinal bacterial fermentation[35,36], which are readily reabsorbed in the large intestine and used by the host as a source of energy. Butyric acid (a SCFA) is known to enhance the cell kinetics of enterocytes[9,37]. In addition, SC-

FAs are also known to play a crucial role in the regulation of intestinal motility, and some of them also protect the host from pathogens[38,39].

Probiotics

"Let food be your medicine and medicine your food", said Hippocrates 400 BC implying that consumption of healthy food would lead to a healthier life. Metchnikoff extended this concept and suggested that by consuming lactobacilli it would be possible to displace pathogens in the GI tract and thereby promote health and prolong life[40]. It has taken many years to identify specific strains that possess such probiotic properties.

A recent definition of probiotics is "Live microorganisms which when administered in adequate amounts confer a health benefit on the host"[41]. Probiotic strains are considered non-pathogenic and safe[42]. The effects and mechanisms of action of probiotics (see below) are far from being completely understood[43,44]. It has been commonly agreed that they exhibit specific properties, which are strain specific and cannot be extrapolated to other strains[45]. Included in the probiotics are several species, but most are lactic acid producing microbes. There are several ways in which probiotics are claimed to have beneficial effects, and some of these have been elucidated recently[141,142]. These includes trengthening of the intestinal barrier, modulation of the immune response and antagonism of pathogens either directly by production of antimicrobial compounds or by competing for binding sites[46,47]. One specific mechanism by which probiotic strains can target pathogens in the GI tract is via production of a bacteriocin (antibacterial protein)[48,49].

Several reviews are available today showing clinical effects when taking probiotics. Randomized controlled trials and meta-analyses support probiotic effects in reducing the risk of diarrhea and shortening it's duration[50,51]. Moreover, the beneficial use of probiotics in prevention of allergic diseases has been demonstrated in studies with administration of LGG (*Lactobacillus rhamnosus*) to breast-feeding mothers and infants of high risk of atopy, which decreased the prevalence of atopic dermatitis after 2 and 4 years[52,53]. Recent studies reported a positive probiotic effect in preventing NEC (necrotizing enterocolitis) by reducing the incidence and severity of this serious disease[54,55]. In yet another study, a combination of different probiotic strains was successfully used to prevent pouchitis in patients with enterostomy[56]. There is already a long research history of probiotics, although the scientific and medical quality requires improvement[57]. Mechanistic basis of their effectiveness needs to be provided and there is a pronounced need for establishment of clear recommendations[58].

Bacterial nitrogen metabolism

In nature

Nitrogen is crucial for all living organisms as an essential part of proteins and nucleic acids. The earth atmosphere contains about 80% nitrogen gas (N_2). As molecular nitrogen is too inert to react with other elements, all multicellular organisms need help to incorporate it. Microorganisms in soil and water initiate the nitrogen cycle by "fixing" the atmospheric nitrogen into the usable form for plants (as ammonia, nitrate or nitrite etc)[59]. Animals consume this nitrogen incorporated in plants and the cycle is completed when the bacteria convert the nitrogen bound in animals or plants back to nitrogen gas. Other nitrogen compounds formed or metabolized by bacteria are HNO_3, NH_3, and the oxides NO, NO_2, N_2O_4, and N_2O.

In humans

Some nitrogenous compounds, such as nitrate, were long considered as potential human health hazards, especially when given to infants and causing the condition known as methemoglobinemia ("blue baby syndrome"). In the gut, nitrate is converted to nitrite, which reacts with hemoglobin to form methemoglobin, thereby decreasing the ability of the blood to carry oxygen. Nitrate was further claimed to be a carcinogenic substance[60,61] via nitrite-mediated formation of nitrosamines. The fear of nitrate has continued to flourish over years despite lack of evidence for a carcinogenic effect in humans. Today, however we are beginning to face somewhat of a para-

digm shift, as nitrate and its metabolite nitrite are also claimed to possess beneficial properties[62-66] (see below).

The utilization of nitrogen sources by the human intestinal bacteria is not fully understood, as the majority of studies have been done with ruminants. However, several similarities exist between intestinal and rumen bacteria. There are several sources of nitrogen in the mammalian intestine. Exogenous sources include dietary proteins and vegetables containing much nitrate[67]. Endogenous sources include excreted cells, and end metabolites such as urea, uric acid, NH_3 and NH_4[68]. N-containing components reaching the colon are proteolysed to amino acids, deaminated to ammonia, CO_2 and branched chain fatty acids[69]. Depending on what compound is formed, they can either be absorbed by the host or excreted in faces.

One of the intermediates of bacterial nitrogen metabolism, nitric oxide (NO), exhibits a variety of biological actions in the gut. Since NO is a main theme of this thesis, a short overview of its chemistry, synthesis and physiology is presented below.

Nitric oxide

A simple molecule of just one atom of oxygen and one of nitrogen, NO was probably generated in the primitive atmosphere. For a long time, nitric oxide was regarded as, at best, an ”ephemeral substance”[70] and, at worst, a poison.

Surprisingly, this common toxic air pollutant formed in car engines and in cigarette smoke, controls an almost limitless range of functions in the body and is well accepted today to be a biological messenger of key importance[71]. NO modulates many physiological functions, including blood flow, respiration and gastrointestinal motility. It acts as a signalling molecule in the nervous system and when produced in large quantities by white blood cells, NO becomes a powerful weapon against invading bacteria and parasites. These functions of NO have been reviewed in detail elsewhere[72-74]. In this thesis, the focus is on bacterial contribution to NO generation in the gastrointestinal tract.

Basic chemistry

NO is a small and easily diffusible gas molecule, able to freely diffuse across cell membranes and activate targets in surrounding cells. Inactivation of NO occurs mainly via oxidation to nitrate and nitrite, e.g. following reaction with proteins for example hemoglobin.

NO is a free radical and as such it reacts readily with e. g molecular oxygen or superoxide (O_2^-), a by-product of both normal cellular metabolism and pathological processes such as inflammation and hypoxia. The reaction product, peroxynitrite ($ONOO^-$), decomposes to hydroxide or nitrogen dioxide radicals, which are much more toxic than NO, particularly affecting DNA and enzymes involved in DNA repair[59,75].

At low and biologically relevant concentrations, NO is surprisingly stable in the gas phase but extremely labile in biological tissue fluids. Due to its chemical reactivity, methods for its measurement have been developed; reacting with ozone, NO generates an excited and therefore chemiluminescent product, NO_2*, which is used to detect gaseous NO.

NO can be “stored” as S-nitrosothiols (SNOs), after reacting with thiols in proteins. Alternatively it can form pools of bioactive NO in blood by oxidation to nitrite[63,76]. The chemistry of NO is complex, and, because NO and its metabolites are highly reactive, it is difficult to determine exactly which molecule enhances certain biological responses (Fig. 2). Therefore many aspects of NO biology still remain to be elucidated.

NO synthase-dependent NO formation

The ability of organisms to produce nitric oxide is an ancient one, developed long before mammals emerged[77]. In mammalian cells NO is synthesized from the amino acid L-arginine and molecular oxygen. This reaction is catalysed by nitric oxide synthases (NOS) in vascular endothelium, smooth muscle, cardiac muscle, and other cell types. Site and onset of NO production from the individual NO synthase isoforms seem to play different roles in different physiological processes[73].

To date, two main categories of NOS are known in mammalian species; constitutive and inducible. Constitutive NO synthase (cNOS), with two isoforms identified in endothelial and neuronal cells are named accordingly, endothelial NOS (eNOS) and neuronal NOS (nNOS). Endothelial NOS and nNOS are activated by increases in intracellular calmodulin and Ca^{2+}, leading to low and transient levels (nanomolar range) of NO. In contrast, the inducible form of NOS (iNOS) is essentially independent of intracellular Ca^{2+}. It is activated via transcription factors stimulated by proinflammatory cytokines or bacterial endotoxins[78]. Induction of iNOS thus requires time for gene upregulation which later results in enormous NO production (micromolar range).

There are both selective and non-selective NOS inhibitors. Aminoguanidine has been proposed to be a selective inhibitor of iNOS[79] but is not very potent. The majority of NOS inhibitors are arginine analogues, like N^G-monomethyl-L-arginine (L-NMMA) and the more potent N^G-nitro-L-arginine methylester (L-NAME)[80]. They inhibit activity of both cNOS and iNOS.

Generation of NO in the human body

L-arginine pathway

$$\text{L-arginine} + O_2 \longrightarrow \text{L-citrulline} + NO$$

Reduction of nitrite under acidic conditions

$$NO_2^- + H^+ \leftrightarrow HNO_2$$
$$2HNO_2 \leftrightarrow N_2O_3 + H_2O$$
$$N_2O_3 \leftrightarrow NO + NO_2$$

Chemistry of NO in the human body

Autoxidation

Gas phase reaction
$$2NO + O_2 \longrightarrow 2NO_2$$

Aqueous reaction
$$4NO + O_2 + 2H_2O \longrightarrow 4NO_2^- + 4H^+$$

Oxidation reactions with

- hemoglobin
$$NO + Hb(Fe^{II})O_2 \longrightarrow NO_3^- + Hb(Fe^{III})$$

- superoxide
$$NO + O_2^- \longrightarrow ONOO^-$$

Figure 2. *Generation and some chemical reactions of NO in the human body.*

NOS-independent NO formation

NO is also formed in the human body by reduction of nitrite ($NO_2^- \rightarrow NO$), as first described in the stomach[66,81]. Ingested nitrate (abundant in green leafy vegetables, e.g. lettuce and spinach) is absorbed from the gut into the blood and concentrated up to 10 times in the salivary glands by an active transport system[67,81,82]. When dietary and endogenous nitrate is excreted by the salivary glands facultative anaerobic bacteria in the oral cavity reduce parts of it nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$)[61]. The acidic environment of the stomach favours spontaneous reduction of nitrite to NO[81]. Besides the acid catalyzed reduction of nitrite, other pathways for NO formation from nitrite have subsequently been described, e.g. enzymatic reduction by xanthine oxidase[83-85], mitochondrial enzymes[86] .

NO generation by bacteria

Some bacteria in soil have the unique capability of enzymatically reducing nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$) as a part of the nitrogen cycle. The reduction of nitrate occurs via two different routes, either through an aerobic pathway, where nitrate is reduced to ammonia followed by incorporation of nitrogen to biomass, or by an anaerobic pathway, where nitrate is reduced to nitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) in the course of respiration. Bacteria with nitrate reductase activity (e.g. staphylococci) colonize the human skin and the GI tract and are present at their highest density in the oral cavity, large intestine [1] and in breast milk[87,88]

This thesis is a part of a rapidly growing field of research suggesting that bacteria inhabiting our body surfaces can contribute to the generation of NO. A picture is now emerging suggesting that nitrogen metabolism by bacteria is not only a "bacterial matter" but also lead to mutual host

microbial benefit[64,65]. This thesis will hopefully contribute to a better understanding of the relationship between the microflora and the host. It is tempting to speculate that nitric oxide might be one of the crucial messengers in this communication.

AIMS OF THE THESIS

The overall aim of the thesis is to investigate whether the intestinal flora contributes to generation of NO in the gut. The specific aims of the work described in the thesis were:

- To develop methods for measurement of luminal NO in neonates and rats, and from bacteria *in vitro*
- To monitor changes of colonic levels of NO in newborn infants during their first days of life
- To determine whether commensal bacteria can generate and/or consume NO
- To characterize factors influencing bacterial generation of NO
- To study *in vivo* generation of NO in the gut of germfree, monocontaminated and conventional rats

Material and methods

For a detailed presentation of materials and methods the reader is referred to the individual papers. All studies were approved by the local ethics committees and all parents to the infants gave informed consent for participating in the studies.

Studies involving newborn infants (Papers I, III and IV)

In **Paper I** and **IV** 60 newborn infants delivered either vaginally or by elective Caesarean section were included. Exclusion criteria were asphyxia at birth, or malformations. All infants were breast-fed on demand from the first hours of life and subsequent feeding practices were monitored throughout the study period. In **Paper I,** luminal NO was sampled and analysed at five occasions: immediately after birth, day one, day 3 - 5, one and 5-6 months after birth. In **Paper IV**, a single measurement of luminal NO was performed at 3 - 6 days after birth.

Faecal samples were collected (**Paper I, III and IV)** in conjunction with the gas measurements and analysed for Short chain fatty acid (SCFAs) pattern (**Paper I).** NO generation from faecal bacteria was detected using chemiluminescence assay (**Paper III)** and nitrate and nitrite levels in faeces from the infants were measured (**Paper IV)**. In addition, *S.aureus, E.Coli, Bifidobacteria* and *Lactobacilli* strains were isolated from the faeces of two healthy newborn infants and these microbes were tested for *in vitro* NO generation and/or consumption (**Paper IV)**.

Animal studies (Papers II, III and IV)

In **Paper II and III**, adult germ-free (GF) AGUS rats[89] were compared to conventional counterparts. These rats were kept in Gustafssons stainless steel isolators and the conventional counterparts in an ordinary animal facility[90]. Both the GF and conventional rats received a steam sterilised rodent diet (Lactamin, Sweden) and water *ad libitum*. The GF animals were inbred for more than 90 generations at the Laboratory of Medical Microbial Ecology and the GF status was checked weekly[91]. In addition to AGUS rats, adult conventional Wistar rats were used (**Paper II and IV)**. The rats in **Paper IV** were given a special low-nitrate diet (TD 99366 chow for rats, Harlan, USA) and kept in an ordinary animal facility. This diet was used to control the nitrate intake. Therefore, rats in this study **(Paper IV**) are not fully comparable to the ones in two previous studies (**Papers II, III).**

Effects of nitrate, NO synthase inhibitor.
To further study bacterial contribution to the intestinal NO production, a nitrate load was given to rats (**Paper II** and **IV**). Either sodium nitrate ($NaNO_3$, 0.1mM/kg) in 1 ml distilled water or the same amount of 0.9 % NaCl was given through a gastric gavage to fasting rats. Nitrate levels were determined using a commercially available kit.
To further study the contribution of mucosal NO synthase to luminal NO, we used the NO synthase inhibitor, L-NAME. Either L-NAME (100mg/kg) or water was given to the fasting rats via gastric gavage (**Paper II**).

Bacteria

The bacterial strains included in these studies were cultured anaerobically at 37°C for 24-72 hours and 100 µl (about 10^9 CFU/ml) of the respective culture was inoculated on different agar plates, with or without supplementation of 0.1mM of $NaNO_2$ or $NaNO_3$.

In a separate measurement setting we performed dose-response experiments with varying amounts of bacteria inoculated on the respective plates and with varying nitrate and nitrite concentrations.

Chemiluminescence assay for NO measurements

In order to analyse gaseous NO, we used a rapid-response chemiluminescence system (Aerocrine AB, Sweden). These measurements are based on NO reaction with excess ozone (O_3) to generate NO_2^* in the excited state. Upon return of NO_2^* to its ground state (NO_2), the excess of energy is released as a photon and light (luminescence) with wavelengths of 640-3000 nm is emitted. The amount of light produced is directly proportional to the concentration of NO, and the intensity of luminescence is converted to an electric signal and displayed as NO level within less than 0.7 seconds. The assay is extremely sensitive, with a detection limit of 1 ppb, exhibiting a linear response to NO concentrations between 1-100000 ppb. Furthermore, the assay is highly specific for NO, without interference from other nitrogen oxides [92] (Fig. 3).

Measurement of luminal NO

Colonic gas measurements in newborn infants
Colonic gas samples were collected using a tonometric balloon technique[93] (**Paper I and IV)**. An all-silicone catheter (Sherwood Medical, Ireland) equipped with an inflatable balloon tip was inserted 10 cm into the sigmoid colon via rectum. The balloon was inflated with 5 ml NO-free air and left in the intestine for 5 min. After the incubation period, the gas was aspirated and immediately injected into a chemiluminescence NO analyzer. While leaving the catheter in place, the same sampling procedure was repeated to measure H_2 gas (**Paper I)**, which was analyzed by an electrochemical sensor device (Bedfont Ltd, UK).

Animal experiments
After anaesthetizing and the following laparotomy, NO-free air was directly inflated into different luminal compartments of the GI tract: the stomach (4 ml), 5 cm of a mid-section of the small intestine (2,5 ml), the caecum (5 ml), and 4 cm of the distal colon (3 ml). External clamps were used to prevent the air from passing into neighboring compartments. The air was incubated for 15 seconds in the above-mentioned compartments and thereafter aspirated and immediately injected into a chemiluminescence analyser to determine the peak NO concentration. Samples with a volume less than 5 ml were diluted to 5 ml before measurement to assure full recovery of the NO signal. The ambient NO was below 10 ppb in all experiments.

The values of NO measured can be used to compare different groups of animals (GF *vs.* conventional or nitrate vs. controls), and we can safely say that basal NO levels are higher in the stomach than in the lower parts of the GI tract. However, we cannot say anything about the absolute values of NO present in the different compartments. Injection of air dilutes NO and the extent of this dilution will also vary with different injection volumes.

Measuring NO production *in vitro*

After incubation of the bacterial strains, 10 ml gas was aspirated from the gas-tight bags and immediately measured with a chemiluminescence system. To study NO consumption, we inoculated *lactobacilli*, *E. coli, bifidobacteria* and *S. aureus* on respective agar plates, and after 16 hrs, NO gas, 1500 ppb was injected into the gas-tight bags. Thereafter, 10 ml of gas sample was aspirated hourly and analyzed as described above during following hours. The method we developed is described in detail in **Paper III.**

Statistical analyses

Statistical analysis and graph plotting were performed with Prism 4.0 (GraphPad Software, San Diego, California, USA). Because of skewed distributions, H_2 and NO data are presented as median (range), whereas SCFA-values are given as mean (SD) (**Paper I**). To analyse any possible influence of delivery mode and postnatal ages, the H_2 and NO concentrations were normalized by log-transformation and compared using Newman-Keuls Multiple Comparison Test and Repeated Measures ANOVA with post-test for linear trend (**Paper I**).

When the number of rats in the groups were less than 10, NO values were given as median and range (**Paper II, IV**) and as median (25 - 75 percentiles), (**Paper III**). Statistical differences between groups were calculated by Mann-Whitney test (**Paper II and IV**) and correlations were analyzed with the Spearman rank test (**Paper III** and **IV**). A P value ≤ 0.05 was considered significant.

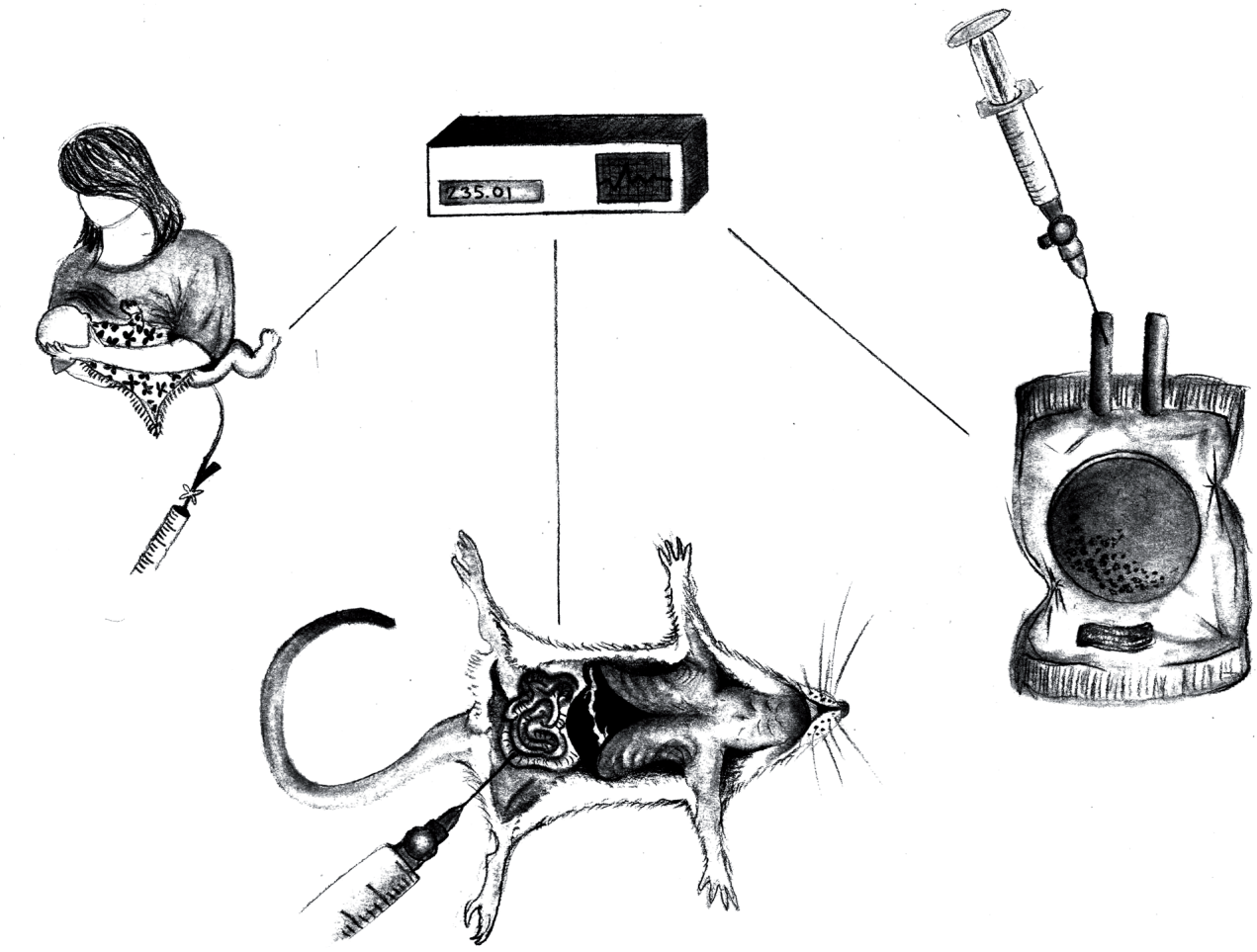

Figure 3. *Overview of the methods used to measure gaseous NO with chemiluminescence: in the colon of a newborn infant, using a tonometric method; in the gut of a rat, by inflating NO-free gas into different intestinal compartments; and from bacteria, grown on plates in gas filled plastic bags*.

Results and comments

For a detailed description of results the reader is referred to the individual papers.

Paper I. Birth-related changes in intra colonic H_2 and NO

First, in this study we wanted to investigate whether NO could be measured in the intestine in the neonatal period. Second, we intended to study whether NO could serve as an early marker of activation of the innate host defence response during the first colonization of the intestine. Knowledge about the response to early colonization is of importance since disturbances in this process are thought to be of relevance for example, in allergy development[94-97]. Third, the knowledge of the normal levels of NO in the intestine of newborns would indicate a possibility of using NO as an inflammatory marker in the gut.

In order to make sure that the procedure would not impose any risks to the neonates several model experiments were made post-mortem in animals. Thus, while placing the balloon catheter in the distal colorectum of rabbits (weight approx. 2000 grams), it was checked, that the insertion and inflation of the balloon catheter did not stretch the gut wall. It was also made certain that all gas used to inflate the balloon could be withdrawn while leaving the catheter in place, before the second inflation was performed for H_2 measurement.

The developed technique for colonic gas sampling was well tolerated by all infants. H_2 and SCFA appeared during the first postnatal days, in parallel with the early bacterial colonization. The earliest and strongest response was in H_2, which, after 24h in vaginally delivered neonates, had reached almost 50% of the levels found in infants at 1-6 months of age. SCFAs reached only modest levels during the first 3-5 days of extra uterine life, and continued to increase over the 6 months observation period. Intra-colonic NO remained very low until 3-5 days after birth and thereafter, NO levels markedly increased. In some apparently healthy infants NO reached levels similar to those seen in adults with inflammatory bowel disease[93,98,99]. Interestingly, there was a significant lagged response in both H_2 and NO in the neonates delivered by Caesarean section, suggesting that there was a delay in colonization of the intestine of these children. On the other hand, in seven children there was a profound increase of colonic NO levels, and six of these infants had been fed a cow's milk based formula. After the first week of life, neither colonic H_2 nor NO levels varied in relation to mode of delivery or postnatal age.

We concluded that the infant groups in this study initially had a slightly different colonization pattern, with a delayed colonization in the Caesarean section delivered group. Our method was minimally invasive and easy to use, and we speculated that intra-colonic gas measurements could be useful to monitor the colonization process as well as mucosal immune activation in a neonatal gut.

Paper II. Gastrointestinal NO in conventional and germ-free animals

In the **Paper I** we observed a high variation of NO levels during the first months after delivery. During this time the infant gut is obviously exposed to a myriad of colonizing bacteria, and we speculated that bacteria themselves could contribute to the colonic NO generation. At the same time, in another study at our lab, colonic NO in patients with collagen colitis was not influenced

Table 1 Levels of colonic nitric oxide (NO), hydrogen gas (H_2) and faecal short-chain fatty acids (SCFAs) in infants delivered vaginally or by Caesarean section.
*= $p<0.05$ for differences between groups

	Postnatal age				
	0-2 hours	24 hours	3-5 days	1 month	5-6 months
NO (ppb)					
Vaginal delivery	8.5 (7.5-17)	11 (6.5-79)	75 (0-7113)	61 (18-721)	110 (14-1590)
Caesarean section	11(5-31)	12 (5-21)	19 (0-3098)	63 (18-1458)	350 (15-1900)
H_2 (ppm)					
Vaginal delivery	<1	47 (1-417) *	429 (1-7000)	373 (8-7000)	523 (19-6000)
Caesarean section	<1	<1	220 ((1-1500)	1778 ((46-15000)	813 (1-5846)
SCFA (mmol/kg)					
Vaginal delivery	5.4±0.6	12±2	44±11	65±10	136±20
Caesarean section	4.1±0.1	8.4±1	21±4	49±10	144±20

by a high systemic dose of NOS inhibitor, indicating that NO could come from other sources than the mucosa [100]. To investigate the bacterial contribution to NO levels in the gut, we performed experiments in GF rats, an animal model which entirely lacks bacteria. We developed a method to measure gaseous NO directly in the lumen of different compartments of the GI tract of GF and conventional rats. We found that in conventional rats, luminal NO differed profoundly along the gastro-intestinal tract with the greatest concentrations in the stomach and caecum (Fig.1). In contrast, in GF rats, NO was low throughout the whole GI tract (Fig. 2). We assessed mucosal NO production by using a NOS inhibitor and showed that the groups did not differ in their NO concentrations in any parts of the GI tract except in the colon where they were significantly lower in L-NAME treated animals.

Finally, to evaluate microbial contribution to the NO generation, nitrate as a possible substrate for bacterial NO generation was given to the animals. After a nitrate load gastric NO increased greatly in conventional but not in GF rats, thereby demonstrating nitrate to be a substrate for bacterial NO generation. After incubating the luminal intestinal contents from the rats pre-treated with nitrate, we measured released NO and observed that the amounts released from caecal contents correlated to the intra-luminal NO levels found *in vivo*, $r = 0.72$.

Our results in this study show that the intestinal microflora indeed is involved in gastrointestinal NO generation. In fact, the presence of bacteria is crucial for intestinal NO generation as measured in the lumen. Another observation was that NO levels exist in the gut lumen, but vary considerably from compartment to compartment (Fig. 4 and 5), reflecting complex host-microflora cross - talks.

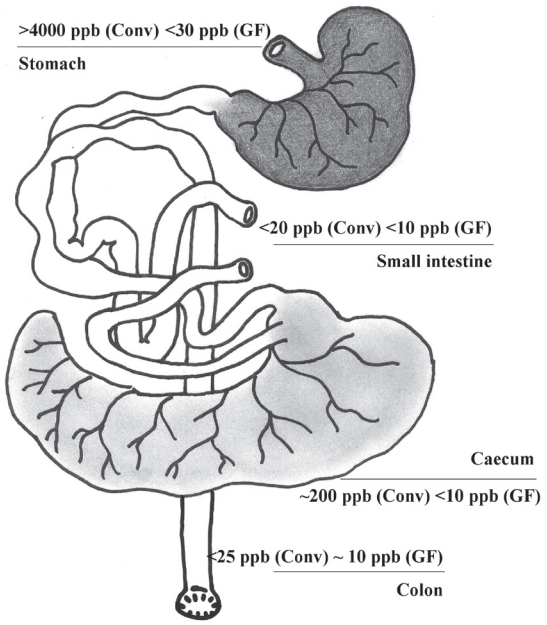

Figure 4. *Nitric oxide levels measured in different parts of the gastro-intestinal tract of conventional non-fasting and GF rats.*

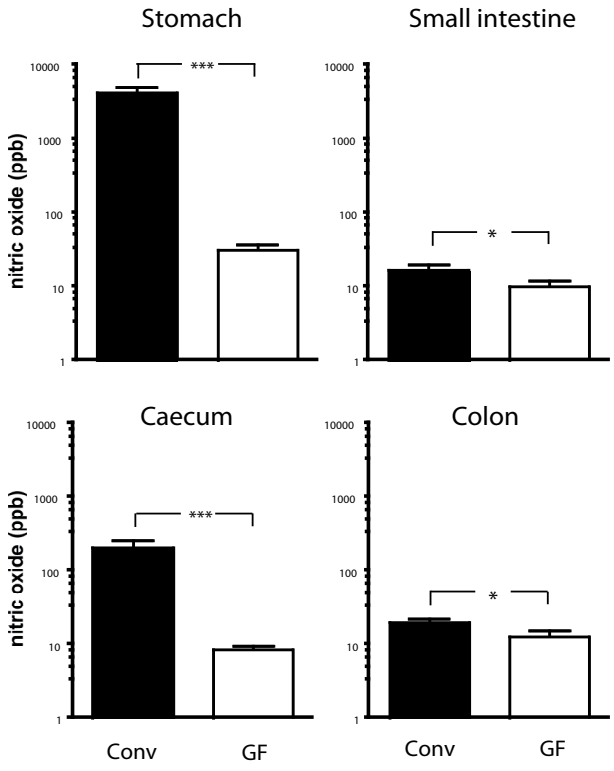

Figure 5. *Intraluminal nitric oxide gas measured in different parts of gastro-intestinal tract of germ-free (GF) and Conventional (Conv) Agus rats. Stomach and caecum (*** p<0,001), small intestine (*p<0,01), colon (*p<0,01) vs. corresponding values in GF animals.*

Paper III. Gastrointestinal bacteria generate NO from nitrate and nitrite.

To understand how nitrate could be involved in intestinal NO generation, we investigated if commensal GI bacteria can directly generate NO from nitrate and nitrite. Of special interest was to study the possibility of lactobacilli and bifidobacteria to form NO, since the literature on the subject suggest that these bacteria might be early members of the flora[9]. Therefore, we developed a method to culture bacteria for measurement of gaseous NO under aerobic and anaerobic conditions. The method allowed us to analyze NO from the cultures at defined intervals. We found that some strains, such as lactic acid producing bacteria (*Lactobacillus acidophilus* and *Lactobacillus casei Shirota*), generated considerable levels of NO from nitrite *in vitro* (>5000 ppb). However, only a few of some other tested strains (*Lactobacillus reuteri* and *Bifidobacterium infantis*) produced NO from nitrate and at much lower levels (<50ppb). This is in contrast to 4 different *E. coli* strains, of which none generated NO levels above 5 ppb. Reduction of nitrite to NO was likely non-enzymatic and was caused by bacterial generation of acid, catalyzing spontaneous decomposition of nitrite in the culture medium to NO. The reactions of non-enzymatic NO generation are as follows (reactions 1 and 2).

$$NO_2^- + H^+ \leftrightarrow HNO_2$$
$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \qquad (1)$$
$$N_2O_3 \leftrightarrow NO + NO_2$$

$$\text{Ascorbic acid} + 2HNO_2 \rightarrow \text{dehydro-ascorbic acid} + 2NO + H_2O \qquad (2)$$

NO generation in the gut lumen was also observed *in vivo* in conventional rats but not in GF rats or in rats mono-associated with *Lactobacillus rhamnosus* ATCC 53103 *(LGG)*. In contrast, when GF rats were colonized with a normal bacterial flora (ex-GF rats), NO in the caecum increased to levels comparable to those in conventional rats (Fig. 6).

In vitro investigations showed that the faecal flora from healthy volunteers generated small amounts (~75 ppb) of NO without supplementa-

tion of nitrite or nitrate to the medium. However, the NO generation was greatly increased when nitrite (~2000 ppb) or nitrate (~900 ppb) was added to the medium, but there was a large inter-individual variation in NO production. In three out of 8 samples we did not find any NO generation after anaerobic incubation, independent of the condition in which they where cultured. The fact that a mixed faecal flora generated NO from nitrate without a concomitant drop in pH suggests that pathways other than acidification of nitrite are involved.

Since it is known that nitrite is spontaneously reduced to NO in an acidic environment[66,81], we tested how much NO was accumulated in the gas-tight bag when nitrite was added to pre-acidified agar plate without the addition of any bacteria. There was a clear correlation between the pH of the agar plate and NO accumulation. Experiments with heat-killed bacteria showed that for all conditions, with or without nitrite or nitrate supplementation, NO levels were low (<5 ppb) after 24 hours. NO accumulation was highly correlated to the amount of bacteria inoculated on the plates, demonstrating thereby the need of live bacteria for NO generation. Similar experiments with increasing concentrations of nitrite and nitrate added to ISO-sensitest agar plates revealed a dose-dependent increase in NO levels.

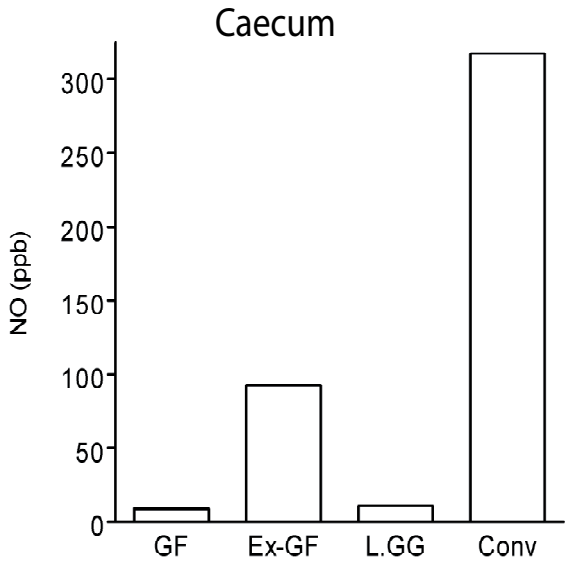

Figure. 6. *NO generation measured in the caecum of Germ-free (GF) rats, rats colonized with a mixed normal bacterial flora for 7 days (Ex-GF), GF rats mono-inoculated with Lactobacillus rhamnosus (LGG) for 7 days, and conventional rats (Conv).*

We concluded that NO can be generated *in vitro* by the anaerobic gut flora in the presence of nitrate or nitrite. Effective NO generation from nitrate seems to require sequential reductions involving different bacteria.

Paper IV. Generation of NO by probiotic bacteria in the GI tract.

To further investigate whether NO can be generated by commensals *in vivo* if substrate is available, we fed rats with live lactobacilli with or without supplementation of nitrate in their drinking water. Dietary supplementation with lactobacilli and nitrate for one week resulted in a 3-8 fold NO increase in the small intestine and caecum, while colon levels remained unchanged (Fig. 7). Caecal NO levels correlated to nitrite concentrations found in luminal contents, ($r = 0.73$, $p = 0.006$).

We also wanted to study how the presence of substrate in breast milk interacts with NO generation during the first days after birth. Thus, we measured nitrite/nitrate in faeces and in breast milk of newborn infants as well as their colonic NO levels. The median of intra-colonic NO levels measured 3-6 days after birth was peaking day 3-4, similar to the results described in the **Paper 1**. These colonic NO levels correlated to the nitrite content of breast milk and in faeces.

From feces of two of the healthy neonates *Lactobacilli sp., E. Coli, Bifidobacterium sp. and S. aureus* were isolated and tested *in vitro* for generation or consumption of NO. The lactobacilli and bifidobacteria generated NO *in vitro*. These findings corroborate the results from **Paper III**. In contrast, when Lactobacilli or Bifidobacteria were co-incubated with *E. Coli* or *S. aureus* there was no NO detected, indicating that the NO generated by the lactic acid-producing

bacteria was consumed by *E. Coli* and *S. aureus* (Fig. 8). Indeed, when exogenous NO gas was added to a gas tight bag with *S. aureus,* the microbes rapidly consumed added NO, reducing the NO levels from 1300 ppb to 2 ppb within less than 8 hours.

Thus, we have found that commensal bacteria can be a significant alternative source of NO in the gut besides the NO produced by the mucosa. Intestinal NO generation can be stimulated by dietary supplementation with substrate and lactobacilli. The generation of NO by some probiotic bacteria can be counteracted by rapid NO consumption by other strains. Future studies will clarify the biological role of the bacteria-derived intestinal NO in health and disease and if an imbalance in generation *vs* consumption has any significance in the pathophysiology of intestinal disorders.

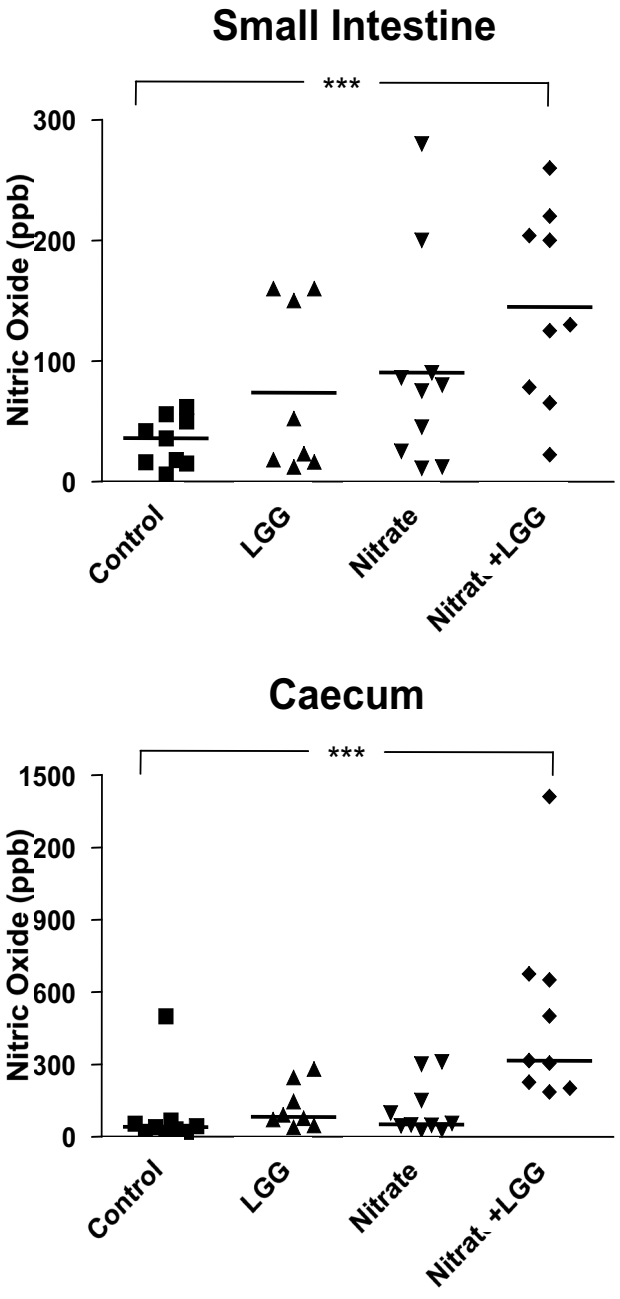

Figure 7. *Nitric oxide levels in the small intestine and caecum of rats fed with Lactobacillus rhamnosus (LGG), $NaNO_3$ 0,1mmol/kg/day (Nitrate), Lactobacillus rhamnosus + nitrate (LGG+Nitrate) and controls (Control). NO was measured directly in luminal gas (ppb).*

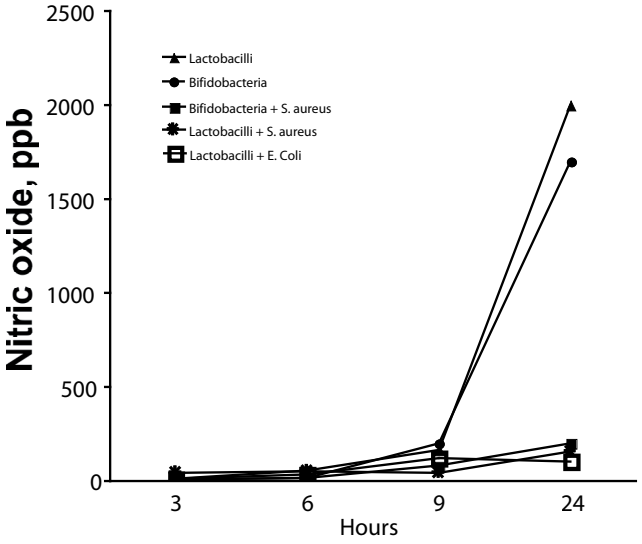

Figure 8. *Generation of nitric oxide by lactobacilli and bifidobacteria inoculated alone or together with S. aureus and E. coli. Bacteria were grown anaerobically on plates placed in gas-tight bags. At indicated time points, a gas sample was drawn from the bag and NO was measured.*

General Discussion

In this thesis we have explored an alternative source of NO in the GI tract – the commensal bacteria. We show that bacteria residing in the gut can generate considerable amounts of NO, and this can be enhanced by the substrates nitrate and nitrite. We also demonstrate that NO generated by some gut bacteria can be effectively consumed by other microbes. Thus, the levels of NO present in the gut reflect the balance between the formation and consumption of NO. A major challenge now will be to elucidate if the metabolism of bioactive nitrogen oxides by gut bacteria plays any role in local and systemic regulation of physiological functions.

Multiple sources of luminal NO generation

One of the questions we tried to answer during this project was whether the NO we measure intraluminally originates from the mucosa or is generated by bacteria. We find this information important, as an induced mucosal NO synthesis by NOSs would signal a possible inflammatory reaction. On the other hand, it is equally important to find out if NO can be generated by bacteria themselves as this could have a physiological impact on the host. During this project we have collected data supporting the existence of both mucosal and bacterial sources of NO.

Mucosal NO generation

Colonic NO was uniformly low immediately after birth and then increased gradually during the first days of life (**Paper I**). In many infants a transient peak in NO on day 3-5 after birth was observed. As these infants seemed to be healthy, this led us to speculate that this was a natural host-response involving induction of NOS in the mucosa in response to the colonizing bacteria. During the first days of life, intestinal H_2 increased in all except one infant. This infant had received intravenous broad-spectrum antibiotic therapy for transient tachypnea at the age of 2 days. Prior to antibiotic therapy at 24 hours of postnatal age, the H_2 level was 308 ppm, reflecting a on-going colonization process. During therapy it dropped to 12 ppm. This obvious decrease in bacteria did not affect NO levels, thereby supporting a mucosal source of NO in this case. However, we are aware of the fact that some strains may be antibiotic resistant; thereby not excluding a bacterial origin of the measured NO. A stronger support for mucosal source of colonic NO is the decrease in NO observed in the rat colon following systemic NOS inhibition with L-NAME (**Paper II**). Finally, when adding LGG and nitrate aiming to stimulate intestinal bacterial NO generation in the rat, the levels of NO were not affected in the colon (**Paper IV**), suggesting other than bacterial sources in this particular part of the GI tract and that a considerable portion of the NO measured in this compartment is of local mucosal origin.

Bacterial contribution

In principle, there are two ways in which bacteria could contribute to intestinal NO production. In the first pathway bacteria stimulate cells in the mucosa to produce NO from a NOS. Indeed, bacterial products (e.g. LPS) are well known efficient inducers of NOS. A second alternative is that bacteria produce NO themselves. Judging from our studies, both mechanisms are likely but they seem to operate at different locations (Fig. 9).

The very high basal NO levels in the stomach, were not affected by L-NAME (**Paper II)**, which suggest predominantly NOS independent generation. Indeed, we here confirm and extend

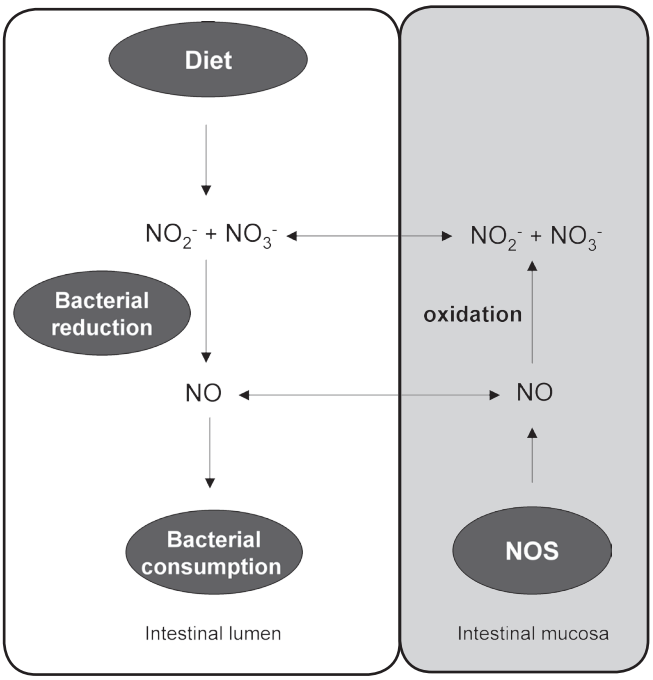

Figure 9. *Schematic overview of different NO sources in the intestinal mucosa and lumen.*

earlier studies[66,81 101] showing that intragastric NO generation is a result of rapid acid-catalysed reduction of salivary-derived nitrite. In this process oral bacteria play a central role since they first reduce salivary nitrate to nitrite. After giving oral nitrate load to rats (**Paper II** and **Paper IV**), more substrate (nitrite) is delivered via saliva and gastric NO increases dramatically. The critical role of bacteria in this process is most clearly illustrated in the experiments with GF rats (**Paper II)**, where gastric NO was uniformly very low and did not even change after a nitrate load.

A central part of our investigations was to study if nitrate supplementation could also increase NO levels further down the GI tract, but for this to happen, some nitrite would have to persist the passage down to these areas. Clearly this does occur *in vivo* as shown in the rat experiments where dietary supplementation with nitrate resulted in increased NO levels in both the small intestine and in the caecum. In contrast, neither GF rats nor the rats mono-inoculated with lactobacilli generated NO even though nitrate was given orally (**Paper III**). The reason for the absence of NO in mono-inoculated animals is probably related to low amounts of substrate (nitrite) entering the area where the bacteria reside. As stated above, this nitrite is derived from specific bacteria residing in the oral cavity. Obviously, such bacteria were not present in the mono-inoculated rats and consequently very little nitrite entered the stomach and the intestines.

While inoculation of GF rats with lactobacilli alone was not sufficient to trigger NO generation, dietary supplementation with the same bacteria to conventional rats resulted in markedly increased NO in the small intestine and the caecum. Similar results were obtained *in vitro,* where most isolated bacteria generated little or no NO from nitrate while a combined fecal flora could generate considerable NO from nitrate (**Paper III**). This suggests that several bacterial species work in concert to generate NO from nitrate. Some species are effective in reducing nitrate to nitrite while others catalyse further reduction of nitrite to NO. Formation of NO by bacteria could occur either via enzymatic reduction of nitrite (bacterial nitrite reductases) or via acid-dependent reduction as seen in the stomach. *In vitro* studies seem to support the latter explanation, as NO formation by lactobacilli was closely correlated

to a pH reduction in the growth medium (**Paper III**). On the other hand, the mixed faecal flora was capable of NO generation from nitrate without large reductions in pH, which may indicate the involvement of enzymatic pathways.

In the colon, NO levels were low independent of intervention. The lack of available substrate may explain this finding, as it is likely that nitrite and nitrate are absorbed or consumed to a large extent before reaching the colon. In our study **(Paper IV)**, neither nitrate nor nitrite increased in the colon after dietary supplementation with nitrate. Alternatively, the number of NO generating bacteria is low in this particular GI compartment. Another possibility is that any generation of NO in the colon is effectively counteracted by a rapid consumption. Indeed, we show that other commensals (*S. aureus* and *E. coli*) can effectively consume the NO generated by lactic acid producing bacteria. Also, NO gas was rapidly consumed when added to *E. coli* or *S. aureus* cultures.

Possible role of dietary nitrate and bacteria in newborn infants

The type of feeding is known to influence both the host and the microflora, especially in the case of newborn infants[102-107]. The newborn infant is exposed to considerable levels of nitrite and nitrate during the first weeks of life as the physiological levels in the breast milk are highest immediately after birth and progressively decline with time[108,109]. The establishing microflora in the newborn infant may directly be involved in the utilization of nitrate in the breast milk, converting it from an inert stable anion that the human cells can not use into the more reactive nitrite. This nitrite could be toxic, but if further converted into nitric oxide, it might have possible physiological effects. In this way, increased amounts of substrate (nitrate) in breast milk might be important for the regulation of bacterial establishment as well as for the donation of nitrogen to the GI tract. The need of nitrogen is known to be higher in newborn infants, as they use it for synthesis of proteins, enzymes etc. Bacteria use nitrogen in the large intestine for de novo synthesis of amino acids. The host can also absorb these amino acids, but this ability is lost progressively with age[110]. The gut as an organ has one of the highest rates of protein synthesis of any tissue in the body, since villous enterocytes are exposed to both the diet and mesenteric arterial circulation. Since the nitrate/nitrite levels in breast milk vary with age and the higher levels might interfere with both NO generation and nitrogen balance in newborn infant, this should probably be taken into account when the infant formulas with constant nitrate/nitrite are introduced early the infant.

Diagnostic aspect of NO measurements

Some inflammatory conditions (e.g asthma and IBD) can be revealed by analysing the luminal levels of NO in the lungs and intestines respectively[99,111,112]. Such tests are attractive since they are non-invasive and provide instant results. Initially, the aim of this project was to study if colonic NO could be safely measured also in newborn infants and if it could be used as a diagnostic marker.

In healthy adults, rectal NO levels are low and quite stable over time[98,100]. As is evident from our study (**Paper I),** this is not the case in newborn infants. Although all infants had almost undetectable NO levels at birth, we noted a marked increase during the first week of life. Importantly, in some apparently healthy infants NO increased to levels similar to those seen in adults with active inflammatory bowel disease. This indicates a vivid stimulation of the intestinal immune system in response to the emerging bacterial flora, a phenomenon seen also with another marker of intestinal inflammation, faecal calprotectin[113-115]. The great variation in normal colonic NO levels early in life indicate that it will be difficult to use intracolonic NO measurements to detect inflammation during this period. In older infants (> 6 months) the normal variability may be lower, which would increase the likehood of detecting alterations in NO generation e.g. as a result of inflammation.

Necrotizing enterocolitis (NEC) is a severe life threatening inflammatory disorder mainly affecting premature infants. Mucosal generation of NO is indeed increased in NEC[116-118], but we don't know if this is reflected in higher luminal NO levels. In preliminary studies we have safely managed to measure luminal NO in healthy premature neonates but the mucosa in NEC cases is severely damaged with increased risk of perforation, which does not allow to use of the sampling method in its present form.

In summary, we have developed a safe and rapid method to directly measure intestinal gases in newborn infants. More studies are clearly needed to evaluate if tonometric gas measurements could be of any diagnostic use in the neonatal period. Nevertheless, the method used here represents a minimally invasive, rapid and reliable way to assess rectal gases in infants. It allows direct sampling in the actual environment of the colon lumen and could therefore be used to study the relationship between the host and the microflora during the early colonization process. Moreover, the method could easily be adapted for measurements of other gases in the colonic lumen.

Possible role of gastrointestinal NO generation

A fundamental remaining issue relates to the role of the NO generated in the gut by probiotics and other commensals. Many mechanisms by which LABs perform their beneficial activity have been described and speculated upon. These include competitive inhibition of the epithelial and mucosal adherence of pathogens and production of antimicrobial substances (H_2O_2, bacteriocins, lactic acid etc)[45,48,119,120]. NO is an extremely potent messenger that regulates vital physiological processes in the pico-/nano molar range. Thus, even minute production of this gas by gut bacteria could be of biological significance. Judging from the known biological properties of NO, it is not unreasonable that some of the positive effects attributed to probiotics can be explained by formation of NO by these bacteria as has been suggested. For the upper part of the GI tract there is clear evidence that nitrite-derived NO affects vital biological processes. Nitrite-derived NO stimulates gastric mucosal blood flow and mucus generation *in vivo*, and *in vitro* studies show that acidified nitrite can effectively kill gut pathogens[64]. The importance of this system in the lower parts of GI tract is currently unresolved. However, it is not unlikely that effects will be revealed in this area as well. NO and other nitrogen oxides generated by lactobacilli from nitrite could help to prevent the establishment of pathogenic bacteria in the gut. Indeed, these reactive nitrogen oxides are highly toxic to many bacteria, including GI pathogens such as *Salmonella, Candida, Shigella, Yersinia* and *E. coli*[64,66,121]. Interestingly, lactobacilli themselves are much more resistant to acidified nitrite[122]. Another small antibacterial molecule released from lactobacilli is hydrogen peroxide. Interestingly, the combined antibacterial effect of hydrogen peroxide and lactic acid generated from lactobacilli is greatly potentiated by the presence of nitrite[74,123], again supporting a role of this anion in modulation of the gut flora by probiotics.

NO generation in the gut may be affected by many factors (Fig. 10) and may, in turn, influence the host. It is tempting to speculate that NO from probiotic bacteria can also affect the host mucosa. Such interactions are well known for other bacterial products e.g. short-chain fatty acids, used by colonocytes as an alternative source of energy[37]. Examples of mucosal functions that theoretically could be modulated by bacteria-derived NO include electrolyte and water transport, gut motility and immunological functions, which are known to be influenced by endogenous NO[124,125]. A recent study by Lamine et al suggests anti-inflammatory effects of NO from lactobacilli[126]. They could reduce experimental colitis with addition of lactobacilli, and the protective effect was abolished when the colon was perfused locally with an NO scavenger (hemoglobin). A possible target for anti-inflammatory effects of NO in the gut could be NF-κB, a transcription factor activated in the intestinal epithelium in response to pathogenic bacteria. NF-κB orchestrates the

inflammatory response e.g. by inducing the expression of proinflammatory cytokines including TNF-α and IL-1. Numerous studies have shown that NO is a potent inhibitor of NF-κB[127,128]. Considering the proximity between the gut bacteria and the epithelial cells, it seems likely to assume that NO can survive diffusion from the gut lumen into a cellular target.

One could also foresee detrimental effects from bacterial metabolism of nitrite in the gut. The classical example is the proposed carcinogenic effects of nitrosamines generated from the reaction of nitrite with amines[129-131]. Since this reaction is greatly accelerated in an acidic environment, most focus has been on the association between nitrate/nitrite intake and gastric cancer. Other potentially deleterious effects of nitrite/NO may include induction of diarrhoea[132] and possibly enhancement of inflammatory reactions[133]. Probiotics have been suggested for use in the neonatal period[43,134,135], but may not always be beneficial and they should be further investigated in more detail, especially when given to infants[57]. The gained knowledge on generation of NO can only give indications of their potential effects as dietary nitrate and nitrite could influence bacterial NO generation. Thus, when administrating probiotics to infants, considerations should be taken to the levels of nitrate/nitrite intake. This applies even to prebiotic bacteria[136,137], as they may contain considerable concentrations of dietary nitrate/nitrite.

NO is not the only nitrogen oxide that can be formed from nitrite. Nitrite may undergo alternative chemical reactions to yield other related compounds. Examples include nitrosation reactions (yielding, for example, highly bioactive S-nitrosothiols)[101] and possibly nitration reactions to yield potentially bioactive nitrated lipids[138]. As nitrite and many of its reaction products also are taken up systemically, there is an intriguing possibility that effects can additionally be seen outside of the GI tract.[63,138,139]. Indeed, recent research shows potent physiological effects of nitrite in the cardiovascular system, for example, in regulation of blood flow and in cytoprotection during ischemia[63,65,140].

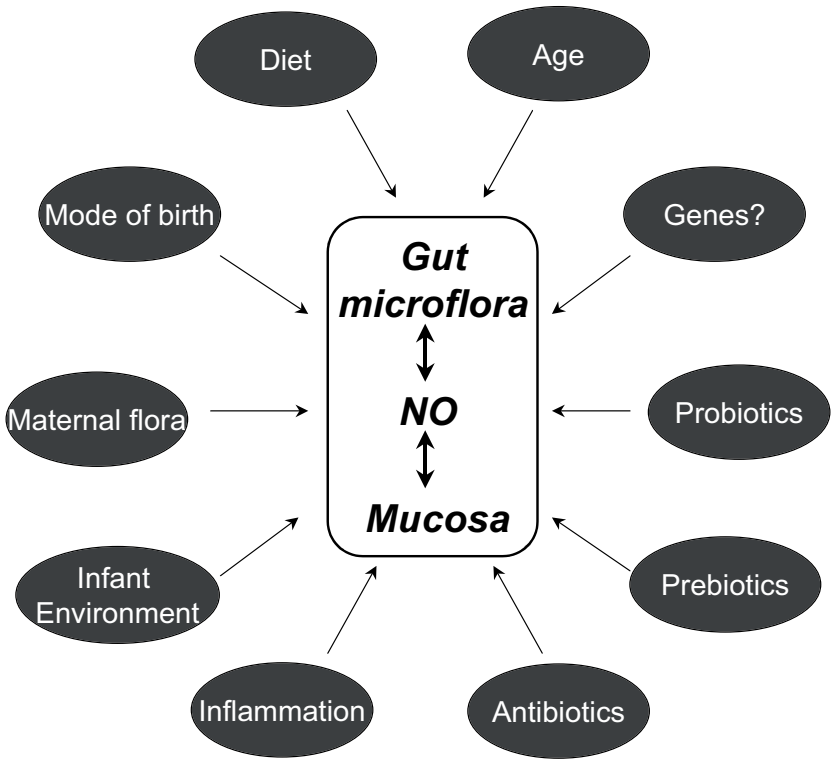

Figure 10. *Factors influencing bacterial colonisation after birth, same factors may also influence NO generation.*

Conclusions

An overall conclusion of the present thesis is that commensal bacteria can contribute to NO generation in the gastrointestinal tract.

Major achievements

- A safe, minimally invasive, rapid and pain-free method for measurements of the intestinal gases H_2 and NO directly in the colon of newborn has been developed
- Intracolonic H_2, SCFAs and NO follow a progression pattern after birth
- NO concentrations in the GI tract are compartmentalised
- The gut commensal bacteria is a significant alternative source of NO in the gut besides the NO produced by the mucosa
- Some commensal strains generate NO from nitrite
- Generation of NO by some probiotic bacteria can be counteracted by rapid NO consumption by other strains
- Intestinal NO generation can be stimulated by dietary supplementation with substrate (nitrate) and lactobacilli

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