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Interactions between bacteria, host and diet in the intestine

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Preface

The intestine is colonized by a vast number of bacteria. In addition, archaea, eukaryotic microorganisms and viruses are present in the digestive tract. The entity of microorganisms that inhabit the gut are referred to as intestinal microbiota. Although this definition includes all microbes, the term microbiota will be used synonymously for the bacterial members throughout this thesis. In a physiological state, gut bacteria beneficially influence host physiology and health. In contrast, alterations in composition and function of the intestinal microbiota have been implicated in adverse effects. Endogenous host factors and nutrition are considered important modulators of intestinal microbiota and may influence the nature of bacterial effects on the host. However, the interactions between gut bacteria, the host and diet are highly complex and far from being completely understood.

This thesis is based on ten peer-reviewed publications that focus on (i) endogenous factors that shape the composition of bacterial communities in a given host, (ii) selected dietary components that affect gut microbiota composition and function, and (iii) the targeted modulation by dietary supplements of intestinal microbiota composition and function. In the **Introduction**, microbiota composition and important functions of gut bacteria are described. **Chapter 1** "**Host factors influencing intestinal microbiota composition"** addresses endogenous factors that may influence intestinal microbiota composition and function including the host genotype and health status. In **Chapter 2 "Interactions between gut bacteria and diet"**, bacterial responses towards dietary intervention in animals with a complex microbiota and in gnotobiotic rodents with a simplified model microbiota are described. In addition, the bacterial transformation of selected dietary ingredients has been studied and effects on host physiology are presented. The efficacy of microbiota manipulation by the use of prebiotics and probiotics, respectively, is the topic of **Chapter 3 "Modulation of intestinal microbiota by dietary intervention"**. Finally, in the **General Discussion** the most significant findings of the studies that are presented in this thesis are discussed in a more general context. In addition, future perspectives for studies on open questions are presented.

INTRODUCTION

The intestine is home to members of all three domains of life including bacteria, archaea and eukarya. However, bacteria contribute the vast majority of microorganisms that colonize the gut. Intestinal bacteria and host mutually affect each other. The nature of these host-microbe interactions can be described as a symbiotic or a commensal relationship (Hooper and Gordon, 2001). The term “symbiosis” refers to a relationship where at least one partner provides a benefit to the other partner without being harmed. Commensal bacteria co-exist with the host without any obvious beneficial or harmful effects. Obligate pathogens are not considered regular members of the gut microbiota. Since it is not always possible to discriminate between a symbiotic and a commensal relationship, the term “commensal bacteria” or “commensals” is used in the following for non-pathogenic bacteria in the gut.

The advent of molecular methods for studying intestinal microbiota at the structural and functional level has significantly increased our knowledge on host-microbe interactions in recent years. However, investigations based on classical culturing methods laid the foundation for a better understanding of the gut microbial ecosystem. For instance, the numbers of total cultivable bacteria in humans, cattle, small ruminants, pigs and birds have been shown to range between 10^{10} to 10^{11} bacterial cells per ml or g of intestinal content (Whitman *et al.*, 1998). Similar numbers have been reported for humans, horses, pigs, rabbits and some ruminants when culture-independent enumeration of gut bacteria was used. Interestingly, the number of total bacteria in the selected animal species was very similar ranging per gram of feces between $\log_{10}$ 11.5 and $\log_{10}$ 11.9 (Furet *et al.*, 2009). The analysis of bacterial 16S rRNA gene sequences in a variety of animal species revealed that the two phyla Firmicutes and Bacteroidetes are dominant in the intestine of mammals. In humans, the dominant Firmicutes mainly belong to the clostridial clusters IV, XIVa and XVI. The Bacteroidetes are individually composed with a high abundance of *Bacteroides thetaiotaomicron*. Members of the Proteobacteria, Actinobacteria, Fusobacteria and Verucomicrobia are present at low levels. Eleven additional phyla were detected, all of which contributed less than 0.5 % to the total intestinal bacteria (Eckburg *et al.*, 2005). Similar to the human situation, the intestinal microbiota in pigs is dominated by Firmicutes including *Eubacterium* and relatives, *Clostridium* and relatives and the *Bacillus-Lactobacillus-Streptococcus* subdivision. Less dominant are members of the *Flexibacter-Cytophaga-Bacteroides* group followed by Proteobacteria, *Sporomusa* and relatives, *Mycoplasma* relatives and Actinobacteria (Leser *et al.*, 2002). The dominance of Firmicutes and Bacteroidetes in pigs was later confirmed. In this study, Clostridiales, unclassified Firmicutes, Bacteroidales, Spirochaetales, unclassified Proteobacteria, and Lactobacilliales were

important members of the intestinal microbiota. At the genus level, *Prevotella* spp. and *Sporobacter* spp. were most frequently identified followed by *Anaerovibrio* spp., *Clostridium* spp. and *Streptococcus* spp. (Lamendella *et al.*, 2011). Firmicutes also represent the most dominant phylum in horses. In this animal species, Verrucomicrobia, Proteobacteria, and Bacteroidetes equally contribute to the total intestinal bacteria (Shepherd *et al.*, 2011).

The application of deep sequencing approaches revealed that the composition of intestinal microbiota is host specific and highly diverse at the species level (Turnbaugh *et al.*, 2009b). The factors involved in the development of individually composed bacterial communities in the gut are poorly identified but it has been proposed that polygenic host traits in conjunction with multiple environmental factors contribute to this phenomenon (Benson *et al.*, 2010). Although the abundance of bacterial species is highly variable, there is some indication that a subset of gut bacteria is shared among many individuals. For instance, more than 1000 intestinal bacterial species have been detected in a metagenomic sequencing study including 124 European study subjects. Each individual harbored at least 160 species. When the gene catalogues were compared, 75 bacterial species were identified that were prevalent in more than 50 % of the study participants; 57 species were found in more than 90 % of individuals. The authors proposed that the number of identified common bacterial species will increase with the availability of more bacterial reference genomes and with increasing sequencing depth of the human gut metagenome (Qin *et al.*, 2010). Moreover, at least two different "enterotypes" based on the relative abundance of *Bacteroides* spp., *Prevotella* spp. and *Ruminococcus* spp. have been identified in human study populations (Arumugam *et al.*, 2011; Wu *et al.*, 2011). The nature of such a "common bacterial core" at the species level requires further investigation. In contrast, it is widely accepted that a considerable number of bacterial genes are widely shared among individuals and the most prevalent genes have been identified. The bacterial genes which can be considered a "core microbiome" are mainly involved in gene transcription and translation processes and in carbohydrate and amino acid metabolism (Turnbaugh *et al.*, 2009b). Functional analyses of bacterial genes that were statistically over-represented ("enriched") in the intestinal microbiome indicate that the metabolism of carbohydrates, amino acids and xenobiotics including the transformation of plant-derived polyphenols belong to the most important bacterial functions in the gut. (Gill *et al.*, 2006). It can be assumed that these genes are important for bacterial survival in the gut and, in addition, encode bacterial functions that influence host physiology. One important example is the fermentation by gut bacteria of undigested dietary material and of host-derived substrates.

Complex carbohydrates contribute the vast majority of substrates for bacterial fermentation. In humans, 70 g – 80 g of these compounds enter the colon (Cummings, 1994). The amount of non-digestible carbohydrates may be higher in farm animals than in humans since mainly

cereal based diets with high concentrations of non-digestible non-starch polysaccharides (NSP) are fed to these animals. For instance, the total NSP content in ileal effluents of pigs can range from 70 g (low-NSP diet) to 200 g (high-NSP diet) per kg feed ingested (Anguita *et al.*, 2006). The principal end products of bacterial carbohydrate fermentation include the short-chain fatty acids (SCFA) acetate, propionate and butyrate. SCFA are rapidly absorbed by the host and it has been estimated that they contribute more than 60 % to the energy requirements in ruminants and 2 % (dogs) to 30 % (pigs) in different monogastric species. In humans, SCFA account for approximately 10 % of the maintenance energy (Stevens and Hume, 1998). In addition to their contribution to the energy supply, SCFA lower the intestinal pH and thereby increase solubility and passive uptake of minerals from the gut. They may also directly influence calcium uptake by increasing the exchange of luminal calcium against cellular H^+ (Roberfroid *et al.*, 2010). Moreover, SCFA have been reported to exert specific effects on host physiology but the exact nature of these effects is not fully understood (Havenaar, 2011). Proteins enter the hindgut to a much lesser extent than carbohydrates and are also utilized by gut bacteria. In addition to SCFA and branched-chain fatty acids, amines, phenols, indoles, thiols, carbon dioxide, hydrogen, and hydrogen sulfide are formed during protein fermentation. Since many of these metabolites may have adverse effects, excessive protein breakdown in the hindgut has been considered disadvantageous (Macfarlane and Macfarlane, 2012). In addition to carbohydrates and proteins, dietary material contains variable amounts of non-nutritive secondary plant metabolites. Polyphenols including flavonoids and lignans represent an important group of these dietary components since they have been claimed to modify sex hormone-dependent processes in the host. In the large intestine, polyphenols undergo bacterial conversion and this conversion is thought to beneficially influence bioavailability and biological efficacy of these substances (Blaut, 2011). Many gut bacteria are able to utilize host-derived substrates such as mucins, which are glycoproteins. One example is the mucin-degrading *Akkermansia muciniphila* which has been isolated from the human gut (Derrien *et al.*, 2004). However, non-digestible dietary components serve as the major substrate source for gut bacteria and fermentation processes in the large intestine essentially depend on the quantity and quality of diet-derived substrates (Macfarlane and Macfarlane, 2012). Therefore, it is likely that nutrition strongly influences the nature of bacterial effects on host physiology and health. The notion that diet influences gut bacteria is supported by the observation that composition and functionality at the metagenenome level of the gut microbiota in 33 mammalian species depends more on the type of nutrition (herbivores, omnivores and carnivores were surveyed in this study) than on the phylogenetic position of the host (Ley *et al.*, 2008; Muegge *et al.*, 2011).

Taken together, it has been demonstrated that (i) intestinal microbiota is complex in its composition and that there is some indication that it is specific for a given host, (ii) that

microbiota composition and function are regulated by diet, and (iii) that interactions between gut bacteria and diet influence host physiology. In the following chapters, studies on selected aspects of these topics will be discussed in more detail. Most of these studies have been performed in rats and inbred mice. The latter animal model provides the advantage that these animals share the same genetic background which should help to reduce variation between the animals. Moreover, genetically modified mice are available that can be used to test whether or not selected host genes or genetically-driven diseases influence diet-bacteria-host interactions. The most striking argument for the use of rodent models is the availability of gnotobiotic animals. Gnotobiotic animals include germ-free animals and animals with a defined intestinal colonization status. Such models have been considered important in nutrition research decades ago (Coates, 1975) and are now extensively used again in obesity research (Backhed *et al.*, 2007; Turnbaugh *et al.*, 2009a). The following specific questions concerning diet-independent and diet-dependent influences on gut microbiota composition and function have been addressed:

1) Does the host genotype influence intestinal microbiota composition in previously germ-free mice under highly controlled experimental conditions? If yes, are the Toll-like receptors TLR2 and TLR4 involved in the selection of a host-specific microbiota?

2) How do intestinal bacteria respond towards chronic gut inflammation? Is it possible to specify bacterial features that provide a selection advantage in the inflamed gut? How do the environmental conditions change under inflammation? Are such changes responsible for changes in microbiota composition?

3) How does the composition of gut microbiota change in response to dietary intervention? Is it possible to develop a model of a simplified intestinal microbiota in gnotobiotic animals? Does this simplified microbiota mimic at large important metabolic functions of complex bacterial communities? Does this model allow the investigation of bacterial responses towards changes in host nutrition at the molecular level?

4) To which extend do bacteria influence the availability of micronutrients (here: selenium) and non-nutritive dietary compounds (here lignans)? Are interactions of gut bacteria with these ingredients relevant for host physiology and health?

5) How effective is the modulation of intestinal microbiota by pre- and probiotics in pigs and in a mouse model of gut inflammation, respectively?

Chapter 1

Host factors influencing intestinal microbiota composition

Diet-independent factors that may influence gut microbiota composition include the genetic background and the health status of the host. The investigation of genetic host factors involved in gut microbiota regulation was the objective of ***PUBLICATION 1*** *"The Toll-Like Receptors TLR2 and TLR4 do not affect the intestinal microbiota composition in mice" (Loh et al., 2008). In addition, the impact of chronic gut inflammation on intestinal microbiota was studied. The differences between healthy mice and mice with chronic gut inflammation in microbiota composition are reported in* ***PUBLICATION 2*** *"Reduced microbial diversity and high numbers of one single Escherichia coli strain in the intestine of colitic mice" (Wohlgemuth et al., 2009). Subsequently, inflammation-associated environmental changes in the gut and their possible impact on the bacterial populations have been addressed. The results of this study are presented and discussed in* ***PUBLICATION 3*** *"Intestinal steroid profiles and microbiota composition in colitic mice" (Wohlgemuth et al., 2011).*

It has been proposed that bacterial populations in the intestine are individually composed and that any given individual harbors a host-specific microbiota (Zoetendal *et al.*, 1998; Turnbaugh *et al.*, 2009b). The mechanisms that contribute to this phenomenon are poorly understood. Studies in twins and less related human study subjects indicated that the genetic background of the host influences intestinal microbiota composition (Zoetendal *et al.*, 2001) but a more recent twin study does not necessarily support this assumption (Turnbaugh *et al.*, 2009b). However, experiments in different inbred mouse strains strongly suggest that the host genotype is involved in the selection of a host-specific set of intestinal bacteria (Friswell *et al.*, 2010). Genetically fixed host factors that may govern gut microbiota composition include the mucin encoding *MUC2*, antimicrobial peptides and proteins produced by Paneth cells in the small intestine and immunoglobulins. Factors involved in antigen presentation such as the major histocompatibility complex and in antigen recognition may also play a role in microbiota control. The latter are mainly represented by pattern recognition receptors including the caspase recruitment domain-containing protein 15 (CARD15, also referred to as nucleotide-binding oligomerization domain-containing protein 2, NOD2) or the various toll-like receptors (TLRs) that sense highly conserved bacterial antigens (Willing *et al.*, 2010). A

broad range of bacterial antigens serve as potential TLR ligands and the molecules that are produced upon TLR activation exert antibacterial effects. Therefore, it can be hypothesized that bacteria that activate TLRs in the intestine are excluded from the gut.

To test the hypothesis that differences in intestinal TLR expression influence intestinal microbiota composition, an experiment was performed in C57BL/10TLR2/4$^{-/-}$ (TLR2/4$^{-/-}$) mice that are simultaneously deficient for TLR2 and TLR4 (PUBLICATION 1). The respective wild-type animals with a C57BL/10 genetic background were used as the control group. To test if genetic factors other than the selected TLRs influence gut microbiota composition under the selected experimental conditions, C3H/HeOuJ and BALB/c mice were also included in this study. All animals were born germ-free (i.e. no bacteria detectable at any site of the organism) and housed in isolators. The mice were colonized with the complex microbiota from one conventionally raised C57BL/10 donor mouse and kept in mixed groups in two different cages in one single isolator. Housing in an isolator was necessary to prevent the colonization of the experimental mice by environmental microorganisms. The housing in mixed groups in two different cages was performed to test whether environmental factors are capable of influencing microbiota composition even under highly controlled conditions. Fecal microbiota development was monitored weekly over 13 weeks with denaturing-gradient gel electrophoresis (DGGE). The mice were killed after this period and selected bacterial groups were quantified in colonic contents with fluorescent *in situ* hybridization (FISH). In addition, short chain fatty acid (SCFA) concentrations in cecal contents were measured. The band patterns in DGGE gels were used as a marker for the fecal microbiota composition and indicated a high microbiota similarity (74.2 ± 8.7 %) when all animals were compared. When the similarities between the animals of the selected strains were compared, C3H mice displayed the highest similarity to each other followed by C57BL/10 TLR2/4$^{-/-}$ mice and BALB/c mice. The highest individuality in microbiota composition was calculated for C57BL/10 wild-type mice. A cluster formation according to microbiota similarity was detected for the mice with a C57BL/10 genetic background on the one hand and C3H and BALB/c mice on the other hand. No effect of the TLR2/TLR4-deficiency was observed. Application of FISH did not reveal significant differences in the cell numbers of bacteria belonging to the dominant groups in the murine intestine including members of the *Eubacterium rectale-Clostridium coccoides* cluster, the Lactobacillus-Enterococcus group and different groups of the Bacteroidetes. Significant differences between mice with a C57BL/10 background and C3H and BALB/c mice in intestinal concentrations of propionic acid, i-valeric acid and valeric acid suggested that bacterial species capable of producing these metabolites were affected by host genotype. The application of a higher number of species-specific oligonucleotide probes and of more sensitive molecular quantification methods might have provided a more detailed picture of a possible host-specific prevalence of the respective bacterial species.

The comparison of the experimental mice that were housed in two different cages revealed that so far unidentified environmental factors have a strong impact on microbiota composition. Interestingly, the “cage effect” was more pronounced in BALB/c than in the other mice. From this study it was concluded that genetic host factors influence intestinal microbiota composition but that TLR2 and TLR4 are not involved in the establishment of a host-specific microbiota.

In addition to genetically fixed host factors, intestinal diseases may influence intestinal microbiota composition. To explore interactions between intestinal microbiota and host under chronic gut inflammation, experiments in interleukin-10-deficient (IL-$10^{-/-}$) mice were conducted. Like many other genetic mouse models of gut inflammation, these mice fail to develop disease in the germ-free state but once the animals are colonized with commensals, chronic colitis evolves (Sellon *et al.*, 1998). The first experiment was set up to characterize microbiota composition in colitic IL-$10^{-/-}$ and healthy control mice (PUBLICATION 2). The animals were born and maintained under highly controlled specific pathogen-free conditions. Five IL-$10^{-/-}$ and control mice, each, were killed 1, 8, 16, and 24 weeks after birth, respectively. No signs of gut inflammation were observed in the wild-type controls. In contrast, IL-$10^{-/-}$ mice developed gut inflammation from week 8 of life on. Severity of inflammation increased with age but never exceeded a moderate level. Lower intestinal microbiota diversity in IL-$10^{-/-}$ than in control mice was deduced from a significantly lower number of bands in DGGE profiles. Two strong bands indicated the presence of two dominant bacterial species in the intestine of IL-$10^{-/-}$ mice. The bands represented *E. coli* (100 % 16S rRNA gene sequence identity) and *Blautia producta* (98 % sequence identity) and it was concluded that these species are highly abundant in the inflamed intestine. The high abundance of *E. coli* in the colitic mice was confirmed with FISH and culture-based methods. Interestingly, numbers of viable *E. coli* increased with age and, thus, with inflammation severity. To better define the role of increased *E. coli* numbers in the inflamed gut, a representative number of *E. coli* isolates from the experimental IL-$10^{-/-}$ and control mice was subjected to phylogenetic characterization. All *E. coli* isolates were members of the virulence-associated phylogenetic group B2. Surprisingly, strain typing and serotyping revealed that the mice were exclusively colonized by one single O7:K1:H7 *E. coli* strain. This strain was tested for the presence of 35 selected genes coding for fitness and/or virulence factors. Twenty of these genes were present in the strain’s genome including genes for adhesins, invasins, protectins, toxins, serine protease autotransporters and iron acquisition systems. In addition, pathogenicity-island markers were detected. The presence of genes coding for adhesins and invasins indicated that the strain was able to adhere to and to invade host cells. However, neither adherence to nor invasion of human epithelial carcinoma cells (Caco-2) was observed. In addition, the strain was not detected in close proximity to the

epithelium when FISH was applied to intestinal tissue material from the experimental IL-10$^{-/-}$ mice. These observations did not provide evidence that the strain was involved in the onset of gut inflammation. However, it can be hypothesized that the high number of virulence- and fitness associated genes provides a selection advantage in the inflamed gut. This notion was supported by the finding that the O7:K1:H7 strain was capable to outnumber other *E. coli* strains from the intestine in competition experiments in previously germ-free mice.

The experiment in IL-10$^{-/-}$ mice suggested that some bacteria have a selection advantage over other species and increase in the inflamed gut. To better characterize environmental factors that possibly exert a selection pressure on gut bacteria, a second experiment in IL-10$^{-/-}$ mice was conducted (PUBLICATION 3). This study focused on alterations in the intestinal sterol metabolism and possible effects of changed sterol concentrations on commensal gut bacteria. Especially changes in bile acid metabolism have the potential to alter microbiota composition since primary bile acids preferentially inhibit the growth of strict anaerobes. In contrast, aero-tolerant Gram-negatives are relatively insensitive (Floch *et al.*, 1972). Therefore, it is possible that a bloom of facultative anaerobic *E. coli* and an increase of bile acid-resistant bacteria is a consequence of inflammation-associated changes in intestinal bile acid metabolism. To test this hypothesis and to investigate if changes in neutral sterol concentrations in the intestine influence gut microbiota, 24 week old IL-10$^{-/-}$ and wild-type mice were used. As expected, IL-10$^{-/-}$ mice developed moderate inflammation of colon and cecum. Moreover, colorectal carcinoma formation was observed in 55 % of these mice. No signs of gut pathology were observed in the control mice. Sequence analysis of bacterial 16S rRNA genes revealed lower intestinal microbiota diversity in IL-10$^{-/-}$ than in control mice. The Firmicutes in the latter were represented by 27 species belonging to 8 bacterial families. In contrast, *Blautia producta, Enterococcus gallinarum, Clostridium innocuum, Robinsoniella peoriensis* and *Lactobacillus murinus* were the only Firmicutes detected in IL-10$^{-/-}$ mice. The relative contribution of Bacteroides to the total microbiota was 25 % higher in wild-type than in IL-10$^{-/-}$ mice. In these animals, both Porphyromonadaceae and Bacteroidaceae were detected. In contrast, no Bacteroidaceae were detected in IL-10$^{-/-}$ mice. In all mice, Proteobacteria were exclusively represented by *E. coli*. The latter contributed a high proportion to the total bacterial sequences in the IL-10$^{-/-}$ but not in wild-type mice. Quantification of gut bacteria with FISH revealed that total bacterial numbers and numbers of members of Bacteroides-Prevotella group were slightly higher in wild-type than in IL-10$^{-/-}$ mice. No differences between the two mouse groups were observed for lactic acid bacteria. Members of the *Eubacterium rectale – Clostridium coccoides* cluster were more often detected in mice with gut inflammation. The largest difference between both groups was observed for *E. coli*, the numbers of which were approximately two orders of magnitude higher in IL-10$^{-/-}$ mice. *E. coli, E. gallinarum, C. innocuum, R. peoriensis* and *L. murinus* were

the most frequently isolated species when intestinal material from IL-10$^{-/-}$ mice was plated on bile-containing agar plates. Total numbers of bile-resistant bacteria did not differ but species diversity was higher in wild-type mice. To find out whether gut inflammation in IL-10$^{-/-}$ mice is associated with an impaired steroid metabolism, neutral steroid and bile acid concentrations were measured in intestinal and fecal material from the experimental mice. Cholesterol and coprostanol represented the neutral sterols. The primary bile acids included cholic acid and α-muricholic acid and the secondary bile acids were deoxycholic acid, 12-keto-deoxycholic acid, and ω-muricholic acid. Cholesterol was the major neutral sterol in the murine intestine. Its bacterial conversion product coprostanol was either absent or detected at very low concentrations. No differences between the IL-10$^{-/-}$ and the control mice in small intestinal concentrations of total sterols were observed and it was concluded that the biliary cholesterol secretion was not affected by the health status of the animals. In contrast, neutral sterol concentrations in large intestine and feces were significantly higher in IL-10$^{-/-}$ mice indicating an impaired sterol uptake or a reduced bacterial sterol transformation in the inflamed gut. A higher proportion of cholesterol relative to coprostanol in the colitic mice suggested a depletion of cholesterol-converting bacteria but high inter-individual differences in the sterol concentrations made it difficult to obtain experimental evidence. High inter-individual differences were also observed for intestinal and fecal bile acid concentrations. The major primary bile acid was cholic acid and the secondary bile acids were essentially represented by deoxycholic acid and omega-muricholic acid. IL-10$^{-/-}$ mice displayed lower concentrations of primary bile acids in the small intestine and of secondary bile acids in cecal and fecal sample material. No differences were observed between colitic and healthy mice in the concentrations of small intestinal secondary bile acid and colonic primary and secondary bile acids. A smaller ratio of primary to secondary bile acids was indicative of a reduced bacterial bile acid conversion in IL-10$^{-/-}$ mice.

In summary, chronic gut inflammation in IL-10$^{-/-}$ mice is associated with significant alterations in intestinal microbiota composition. These alterations are characterized by a bloom of *E. coli* (here: only one single strain) with a high number of virulence- and/or fitness-associated traits. It is likely that these traits support the growth of the strain under hostile environmental conditions in the inflamed gut but they may also provide a selection advantage over other strains in the healthy gut. Among other possible environmental changes, chronic gut inflammation is associated with altered intestinal steroid profiles. Increased concentrations of secondary bile acids may be responsible for changes in gut bacterial community structures. To which extent changes in microbiota composition and/or environmental conditions (e.g. intestinal sterol concentrations) directly influence host health remains to be clarified.

Chapter 2

Interactions between gut bacteria and diet

Responses of intestinal bacteria towards high-fat diets and the consequences for the host have been studied in a mouse model of diet-induced obesity and the results are presented in ***PUBLICATION 4*** *"Absence of intestinal microbiota does not protect mice from diet-induced obesity" (Fleissner et al., 2010). In* ***PUBLICATION 5*** *"Human intestinal microbiota: characterization of a simplified and stable gnotobiotic rat model" (Becker et al., 2011), effects of different diets on the composition of a reductionist model of human microbiota in gnotobiotic rats are reported. Bacterial responses towards selected dietary factors at the cellular level have been studied in E. coli monoassociated mice. The results of this study are presented and discussed in* ***PUBLICATION 6*** *"Impact of nutritional factors on the proteome of intestinal E. coli: induction of OxyR-dependent proteins AhpF and Dps by a lactose-rich diet" (Rothe et al., 2012). Interactions between gut bacteria and dietary components with implications for host physiology and health are the topic of* ***PUBLICATION 7*** *"The gastrointestinal microbiota affects the selenium status and selenoprotein expression in mice" and of* ***PUBLICATION 8*** *"Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model" (Mabrok et al., 2012).*

Intestinal bacteria have been implicated in diet-induced obesity. One of the pioneering studies in this field demonstrated that germ-free but not conventional mice on a high-fat diet are protected from obesity development (Backhed *et al.*, 2004). The underlying mechanisms have not been identified so far and it is still unclear if changes in gut microbiota composition and function are cause or consequence of host obesity. To investigate in more detail the role of interactions between gut bacteria, diet and the host in obesity development, experiments in mice were conducted (PUBLICATION 4). In a first experiment, germ-free and conventional mice were switched from a commercial rodent diet to self-made semi-synthetic diets with a high (43 % energy from fat, HFD) and a low (17 % energy from fat; LFD) fat content, respectively. During the 4 weeks of dietary intervention, body weight, feed consumption and energy intake as well as feed and energy digestibility were determined. In addition, fecal material was sampled before start and after intervention for the enumeration of selected bacterial groups with fluorescence in situ hybridization (FISH). A second animal experiment was essentially carried out as described above but a commercially available high-fat diet (WD) was used instead of HFD. This WD represents a typical Western-style macronutrient

composition with 41 % energy from fat and carbohydrates, each. The most important finding of this study was that germ-free mice are not generally protected from diet-induced obesity. The germ-free animals gained more body weight and body fat than the conventional mice when HFD was fed. Interestingly, the germ-free mice gained less body weight and fat than their conventional counterparts when WD was fed in the second experiment. Since HFD and WD were very similar in their crude fat and carbohydrate contents but differed in the types (e.g. fatty acid composition) and sources (e.g. plant oils in HFD vs. beef tallow in WD) of macronutrients, it was concluded that the type of diet strongly influences the extent of a bacterial contribution to obesity development. To identify bacterial groups that are affected by the differences in dietary composition, FISH coupled with flow cytometry was applied to fecal material which has been collected from the conventional mice. With this method the proportion of selected bacterial groups relative to the total intestinal bacteria can be determined. Before dietary intervention, it was possible to assign approximately 75 % to 80 % of total fecal bacteria to the Firmicutes (represented by the *Eubacterium rectale-Clostridium coccoides* cluster and the *Lactobacillus-Entercoccus* group) and to the Bacteroidetes (represented by the *Bacteroides-Prevotella* group). This proportion dropped to 52 % when the LFD and to 27 % when the HFD were fed in the first experiment. In the second experiment the selected bacterial groups contributed again 27 % to the total bacteria when the HFD was fed but only 19 % when the mice consumed the WD. These observations were indicative of a shift in microbiota composition: bacteria that were detectable by the applied method decreased at the expense of non-detectable groups. In order to better characterize the responses towards dietary intervention of the murine microbiota, a third experiment was conducted with conventional mice. These mice were fed standard chow, HFD and WD, respectively, for 4 weeks. Fecal material was collected for the determination of fecal SCFA concentration and colon content was sampled for microbiota analysis based on 16S rRNA gene sequencing. Independent of dietary treatment, the intestinal microbiota of the experimental mice was dominated by Firmicutes which contributed more than 80 % to the total bacterial 16S rRNA gene sequences. Within the Firmicutes phylum, the proportion of Erysipelotrichiaceae increased from less than 7 % when the chow was fed to 49 % and 21 % when the animals consumed HFD and WD, respectively. The microbiota of mice fed the high-fat diets also contained Proteobacteria and Deferribacteriaceae, which were absent from chow-fed mice. Fecal SCFA concentrations of mice fed the energy-rich diets did not differ considerably. Concentrations in these mice were significantly lower than in mice fed the chow, which is rich in fermentable substrates. Taken together, this study clearly demonstrated that gut bacteria influence the extent of body weight gain on high-fat diets. The notion that germ-free mice are generally protected against diet-induced obesity was not

supported. Specific effects of different high-fat diets indicate that the composition of diet in terms of fat and carbohydrate quality affects intestinal microbiota composition and function.

The experiments with different high-fat diets clearly demonstrated that diet influences intestinal microbiota composition and metabolic activity. However, the exact role of intestinal microbiota in obesity development and specific responses of gut bacteria towards dietary intervention remains elusive. The same is true for the dietary ingredients that are responsible for the observed effects on the microbiota. It has also to be taken into consideration that findings in mice do not necessarily reflect the situation in other mammals. One strategy to overcome the limitation that the intestinal microbiota in mice differs from that in "target species" is the use of germ-free animals that can be colonized with gut bacteria from the species of interest. For instance, mice with a pig-derived intestinal microbiota (Hirayama, 1999) or with a human intestinal microbiota (Hirayama *et al.*, 1995) can be generated. The latter model has been used to study effects of dietary intervention on intestinal microbiota composition and metabolic activities (Kleessen *et al.*, 2001) or the transformation of flavonoids (Hanske *et al.*, 2010; Hanske *et al.*, 2012). These studies demonstrated that it is possible to study interactions between dietary ingredients, gut bacteria and the host taking advantage of rodent animal models. However, the disadvantages of this approach include the fact that microbiota complexity makes it difficult to study such interactions at the molecular level. Therefore, a gnotobiotic animal model with a simplified intestinal microbiota was developed. The bacterial community was composed of *Anaerostipes caccae*, *Bacteroides thetaiotaomicron*, *Bifidobacterium longum*, *Blautia producta*, *Clostridium ramosum*, *Escherichia coli* and *Lactobacillus plantarum* since it was designed to represent numerically dominant bacteria and important bacterial functions in the intestine. At a later stage of model development, *C. butyricum* was added to the consortium (PUBLICATION 5). The bacterial consortium formed a stable community in the intestine of previously germ-free rats and was naturally transmitted from the first generation to the offspring. It was also possible to store the community as a frozen stock and to associate germ-free animals with this inoculum. *B. thetaiotaomicron* was the dominant member of the community (38 % of total bacteria) followed by *B. producta* (28 %), *B. longum* (17 %), *C. ramosus* (12 %), *A. caccae* (5 %) and E. coli (2 %). *L. plantarum* was present at the lowest cell numbers and contributed only 0.03 % to the total bacterial numbers. The simplified microbiota produced acetate, propionate and butyrate at a molar ratio of 84:12:4 in cecum and colon. Since the butyrate production was lower than expected, *C. butyricum* was added to the bacterial community. This modification did not result in an increase of total bacteria but in a repression of other consortium members: cell numbers of *B. longum*, *B. producta* and *A. caccae* decreased by $\log_{10} 3$, $\log_{10} 2$ and $\log_{10} 1$, respectively. Although the butyrate production was increased by more than 50 % by the addition of *C. butyricum*, the concentrations were still much lower

than in conventional rats. Subsequently, it was studied to which extent the model fulfilled other so-called microbiota-associated characteristics than SCFA production. It turned out that the simplified microbiota was able to degrade mucins and ß-aspartylglycine and to convert bilirubin to urobilirubin. These functions were detected in conventional but not in germ-free rats. Inactivation of trypsin by complex rat microbiota was observed but this function was not detected in the bacterial model community. To investigate responses towards dietary interventions of the simplified intestinal microbiota, SIHUMI rats were switched from a standard rat chow to semi-synthetic diets containing either a low amount of fermentable fiber or 10 % inulin or 10 % pectin, respectively. The fermentable fiber material was added at the expense of starch. Feeding the fiber-poor diet instead of the complex standard chow resulted in a significant decrease of total bacteria. The strongest decrease was observed for *B. longum* (reduction by $\log_{10}$ 2.5) followed by *B. producta* (reduction by $\log_{10}$ 1). The numbers of the other bacterial species changed only slightly. Inulin and pectin, respectively, were added to the fiber-poor diet to increase the amount of fermentable substrates. This modification of diet resulted in minor changes in cell numbers. In an additional dietary intervention experiment, a commercially available high-fat low-fiber diet was fed. The fiber-poor diet mentioned above served as a low-fat control diet. In this experiment, numbers of *C. ramosum* were significantly higher in rats fed the high-fat than the low-fat diet. Since *C. ramosum* belongs to the Erysipelotrichiaceae, this finding supports the notion that members of this bacterial group are involved in diet-bacteria-host interactions and possibly in the development of diet induced obesity (see PUBLICATION 4).

It has been demonstrated in conventional (PUBLICATION 4) and simplified animal models (PUBLICATION 5) that intestinal microbiota composition changes in response to dietary composition. However, the mechanisms that enable bacteria to cope with alterations in substrate availability have poorly been investigated. To better understand bacterial adaptation to diet at the cellular level, an animal experiment was conducted in monoassociated mice (PUBLICATION 6). The mice were monoassociated with the *E. coli* K-12 laboratory strain MG1655 and allocated to one of 3 experimental groups. The animals were fed semi-synthetic diets that were composed to permit the growth of *E. coli* (i) on host-derived endogenous substrates, (ii) on dietary carbohydrates and (iii) on dietary protein. The first diet contained mainly highly digestible components (sucrose, starch, casein, sunflower oil) which were thought to be readily absorbed in the small intestine. This diet was used as the reference diet. In the second diet, 10 % of the starch was replaced by lactose which is indigestible to adult mice but can be fermented by *E. coli*. The third diet contained 60 % casein, which was expected to result in high protein concentrations in the large intestine. The mice were killed after three weeks of dietary intervention and intestinal contents were collected for the measurement of substrate concentrations and for *E. coli* enumeration. In

addition, *E. coli* cells were harvested from the intestine and prepared for proteome analysis with 2D difference in-gel electrophoresis (2D-DIGE). Proteins of interest were identified by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry after tryptic in-gel digestion. It was demonstrated by the measurement of glucose, lactose and free amino acids in intestinal contents that it was possible to provide the selected substrates for *E. coli* metabolism at sufficiently high levels. Independent of diet, numbers of *E. coli* were higher in the cecum and colon than in the small intestine. Feeding the lactose-rich diet resulted in higher numbers of intestinal *E. coli* than in mice fed the starch-rich or the protein-rich diet, respectively. Differences in substrate availability resulted in the differential expression of 102 *E. coli* proteins. The most interesting changes were observed when the lactose-rich diet was fed and, therefore, these findings are reported in the following. The availability of lactose (a disaccharide composed of galactose and glucose) caused an elevated expression of enzymes belonging to the Leloir pathway for the metabolism of galactose including galactose mutarotase, galactokinase, galactose-1-phosphate uridyltransferase, and UDP-glucose-4-epimerase. This observation was taken as a proof that the experimental concept was appropriate to study *E. coli* responses to different diets. Interestingly, the ferric uptake regulatory protein (Fur), the alkyl hydroperoxide reductase subunit F (AhpF) and the DNA protection during starvation protein (Dps) were also upregulated when lactose was present in the intestine. The expression of the corresponding genes is under the control of the OxyR transcriptional regulator which controls defense genes protecting *E. coli* from oxidative stress. This activation by a lactose-rich diet of defense mechanisms against oxidative stress was not expected.

To investigate this phenomenon in more detail, the induction of *ahpCF* and *dps* gene expression by glucose, lactose, sucrose and sorbitol was tested in vitro with a luciferase reporter gene assay under aerobic and anaerobic conditions. When H_2O_2 was added as a positive control, an increase in luminescence was observed under aerobic but not under anaerobic conditions. Independent of the presence of oxygen, high concentrations of carbohydrates in the medium increased the level of luminescence. The same was observed when NaCl was added to the medium to increase osmolality. Induction of *ahpCF* and *dps* expression by *E. coli* was no longer observed when the *OxyR* gene was deleted. Therefore, it was concluded that the increased *ahpCF* and *dps* expression under the selected experimental conditions was clearly OxyR-dependent. A correlation between the *ahpCF* and *dps* promoter activities and the osmolality of the media used in the luciferase reporter gene assay was observed. Based on these findings, it was hypothesized that OxyR-regulated gene expression is involved in responses towards osmotic stress. To test whether the genes controlled by OxyR provide a selection advantage under osmotic stress, *ahpCF* and *OxyR* deletion mutants were generated and grown under conditions of high osmolality. Under both

aerobic and anaerobic conditions, the mutants grew to lower cell densities than the wild-type control strain when osmotic stress was induced suggesting that OxyR-regulated genes are protective under conditions of osmotic stress.

In summary, intestinal bacteria respond to changes in dietary composition and substrate availability. The adaptation processes include the differential expression of proteins that are required for the utilization of the respective substrates. In addition, dietary ingredients can influence environmental conditions in the intestine. Gut bacteria have to cope with these challenges and to activate the respective defense systems. The study described above clearly demonstrates that it is possible to study diet-bacteria interactions at the molecular level. However, the use of mice monoassociated with *E. coli* suffers from important limitations. First, *E. coli* does not reach high cell numbers in the healthy gut and it may well be that responses of less important members of the intestinal microbiota are of no or little relevance for host physiology. Second, competition between bacteria for substrates and cross-feeding mechanisms are excluded in monoassociated mice. These bacteria-bacteria interactions may significantly influence the effects of dietary components on bacterial protein expression. Furthermore, the lactose-rich diet was specifically selected to induce expected *E. coli* responses, but it has to be considered that high-lactose diets are usually not relevant in nutrition research. Therefore, it is an important task to modify this model to study dominant gut bacteria in animals harboring a more complex microbiota and to use more relevant dietary ingredients.

A set of experiments with gnotobiotic animals was performed to study to which extent interactions between gut bacteria and dietary components can affect host physiology and health. To investigate possible bacterial effects on host selenium requirement or selenium availability, an experiment with germ-free and conventional mice was conducted (PUBLICATION 7). Directly after weaning, the mice were fed selenium-adequate and selenium-deficient diets, respectively, for five weeks. Blood was collected and the mice were killed at the end of the intervention period. Liver and tissue material from the proximal and distal jejunum, ileum, colon and cecum were sampled. The plasma and tissue selenium concentrations reflected the dietary selenium supply since lower concentrations were measured in mice fed the selenium-poor diet. Germ-free mice had lower cecal selenium concentrations than conventional mice when the selenium supply was normal. When the selenium-poor diet was fed, selenium concentrations in plasma and liver samples from germ-free mice were higher than in their conventional counterparts. From these findings it was concluded that gut bacteria compete with the host for selenium when the supply is limited. To clarify the impact of bacterial selenium sequestration on host physiology, the expression levels at mRNA and protein level of the selenoproteins glutathione peroxidase (GPx) 1 and GPx2 were determined. According to tissue-specific expression or relevance, GPx1

expression was measured in liver and GPx2 expression was determined in intestinal tissue material. Total GPx and thioredoxin reductase (TrxR) activity was measured in plasma (GPx), liver and intestinal tissue material (GPx and TrxR). Independent of the colonization status, gene and protein expression levels and enzyme activities were higher in mice with normal than with low dietary selenium supply. Under selenium-limited conditions, liver GPx1 mRNA and protein expression was higher in germ-free than in conventional mice. The same was true for GPx2 protein expression in jejunum and colon. The higher expression levels in germ-free animals were in line with higher total GPx activities in liver, proximal jejunum, cecum and colon. Higher TrxR activities in germ-free than in conventional mice were detected in all samples tested. It can therefore be deduced that gut bacteria and the host compete for dietary selenium when the supply is low. This competition influences important enzyme systems in the host.

In addition to macro- and micro-nutrients, non-nutritive plant compounds are ingested with diet. The transformation of these secondary plant metabolites including lignans is a critical feature of intestinal bacteria (Blaut, 2011). The majority of lignans is consumed with whole grain cereals, vegetables and fruits but flaxseed is one of the richest sources of the lignan secoisolariciresinol diglucoside (SDG). Epidemiologic and experimental studies suggest that the products of bacterial SDG transformation enterodiol (ED) and enterolactone (EL) are the bioactive compounds (Adlercreutz, 2007). The bacterial conversion of SDG starts with the deglucosylation to secoisolariciresinol (SECO) followed by the *O*-demethylation and dehydroxylation of SECO to ED. The final step is the dehydrogenation of ED resulting in the formation of EL. Under in vitro conditions, the deglucosylation of SDG to SECO is catalyzed by a broad range of intestinal bacteria including different *Bacteroides* spp. and *Clostridium* spp. However, when compared to other gut bacterial species, *Clostridium saccharogumia* displayed a particularly high transformation rate. *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, *Eubacterium limosum* and *Blautia producta* have been shown to *O*-demethylate SECO. The resulting intermediate is dehydroxylated to ED by *Eggerthella lenta* and *Clostridium scindens*. The only so far described species that catalyzes the final dehydrogenation of ED to EL is *Lactonifactor longoviformis* (Clavel *et al.*, 2006). The transformation of lignans by a lignan-converting bacterial consortium was tested in gnotobiotic rats. These rats were associated with *C. saccharogumia*, *B. producta*, *E. lenta* and *L. longoviformis*. When the rats were fed a lignan-rich flaxseed diet, ED and EL production from SECO was observed in the associated but not in germ-free control rats (Woting *et al.*, 2010). Lignan consumption is thought to exert protective effects against breast cancer and it has been proposed that ED and EL are the active compounds. The gnotobiotic rat model exclusively colonized by lignan-transforming bacterial consortium offered the opportunity to investigate the role of bacterial lignan transformation in more detail. To study

the effect of bacterial ED and EL production on breast cancer formation and on selected cancer-associated parameters, three week old female germ-free rats were used (PUBLICATION 8). The animals in the experimental group were associated with the lignan-converting bacterial consortium (LCC rats). The control rats remained germ-free. Two weeks after association of the LCC rats, all animals were switched from standard rat chow to a purified flaxseed diet. Subsequently, breast cancer was induced with a single oral application of 7,12-dimethylbenz(a)anthracene. Thirteen weeks after cancer induction, the animals were housed for 24 h in metabolic cages to quantitatively collect feces and urine for the measurement of lignan excretion. Blood was collected under anesthesia from the retrobulbar venous plexus and plasma and serum were prepared. The rats were killed and intestinal material was sampled. Bacterial cells were enumerated and short-chain fatty acids (SCFA) were analyzed. The latter was performed to exclude that the bacterial production of SCFA from undigested feed material was a confounding factor in this study because these fermentation products have been reported to influence stress-associated signal pathways in human breast cancer cells (Yonezawa *et al.*, 2007). However, SCFA concentrations were comparable in all animals and, therefore, not considered to influence the study outcome. Breast tumors per rat were counted, carefully removed and tumor size and weight were determined. Tumor quality was examined histologically and proliferating (Ki-67 index) and apoptotic tumor cells (active caspase-3) as well as the estrogen receptor status were determined. As expected, all members of the lignan-converting consortium successfully colonized the LCC rats and no bacterial contamination was detected in the control rats. In line with previous observations in this model, conversion of SDG to ED and EL and urinary enterolignan excretion was exclusively observed in the LCC rats. No differences in breast cancer incidence between the experimental and the control group were observed. In addition, the quality of breast tumors did not differ between the experimental and control animals since all tumors were estrogen receptor-positive tubolopapillary adenocarcinomas. However, the number of tumors per tumor-bearing rat and the mean tumor size were significantly reduced in the LCC rats. The quantification of Ki-67- and caspase-3-positive cells indicated that the reduced tumor burden in LCC rats resulted from a lower mitotic activity of the tumor cells in conjunction with higher apoptotic rate. To test whether bacterial lignan transformation influences estrogen-sensitive processes, serum estrogen levels in the experimental and control rats and the expression of the estrogen receptors ERα, ERβ and GPR30 in breast tumors were measured. However, no differences between the LCC and control rats were observed. The same was true for selected genes involved in cell growth, namely *epidermal growth factor receptor* and *insulin-like growth factor 1*. In contrast, activities in liver and plasma of catalase, superoxide dismutase and glutathione-S-transferase all of which are involved in the breakdown of oxidants were

significantly higher in LCC than in control rats. The elevated enzyme activities were indicative of a higher potential of the LCC rats to reduce oxidative stress. However, this assumption was not supported by the measurement of the oxidative stress markers reduced glutathione and malondialdehyde the concentrations of which were equal in all liver and plasma samples. Taken together, this study revealed that the bacterial conversion of SDG to ED and EL does not influence breast cancer incidence but lowers tumor burden in a gnotobiotic rat model of breast cancer. The observed effects in LCC rats cannot be explained by estrogen-dependent mechanisms because estrogen receptor expression and expression of estrogen-sensitive genes as well as circulating estrogen concentrations did not differ between LCC and control rats. There was some indication that enterolignans produced by gut bacteria improve the capacity of the host to degrade oxidants. However, similar concentrations of oxidative stress markers in LCC and control rats did not support this assumption. In conclusion, the transformation by a well-defined bacterial consortium of the plant lignan SDG to the enterolignans ED and EL is the prerequisite for the anti-cancer effects of lignans in this model. The mechanisms involved in the health-promoting effects of enterolignans require further investigation.

Chapter 3

Targeted modulation of intestinal microbiota by dietary intervention

The most widely accepted concepts of targeted microbiota modulation include the use of prebiotics and probiotics. ***PUBLICATION 9*** *"Inulin alters the intestinal microbiota and short chain fatty acid concentrations in growing pigs regardless of their basal diet" (Loh et al., 2006) addresses the potential benefits of prebiotics in pigs. In addition, possible effects of a probiotic bacterium in the interleukin-10-deficient mouse model of chronic gut inflammation have been investigated. The outcome of this study is reported in* ***PUBLICATION 10*** *"Enterococcus faecium NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation" (Ganesh et al., 2012*).

Commensal gut bacteria contribute to host health and well-being but they have also been implicated in intestinal disorders. Therefore, strategies to modify intestinal microbiota composition and function, including the use of prebiotics and probiotics, have been developed. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon" (Gibson and Roberfroid, 1995). This definition includes a large number of dietary ingredients that potentially fulfill these requirements. However, established prebiotics mainly belong to the oligosaccharides and most research has been carried out with inulin-type fructans (Gibson *et al.*, 2010). The application of the latter to pigs may reduce weaning-associated detrimental changes in microbiota composition, improve nutrient utilization and, thus, performance, stimulate immune functions and beneficially influence gut epithelial barrier function. However, beneficial effects of prebiotic fructans in pigs are not always observed (Verdonk *et al.*, 2005). To test the hypothesis that effects of prebiotics are masked by non-digestible carbohydrates in cereal-based pig diets, a feeding trial with growing pigs was conducted (PUBLICATION 9). The animals were fed either cereal-based or semi-synthetic diets. All types of diet were used with and without 3 % inulin, respectively. Feeding the different diets did not influence animal feed intake or body mass gain throughout the study. Eight animals per group were killed after intervention periods of three and six weeks respectively, and jejunal, ileal, and colonic material was sampled. Total bacteria, members of the *Clostridium lituseburense* and the *Clostridium histolyticum* group,

the *Eubacterium rectale-Clostridium coccoides* cluster, *Bacteroides* spp., bifidobacteria, lactobacilli, enterococci and enterobacteria were enumerated with fluorescent in situ hybridization (FISH) in the intestinal contents. In addition, concentrations of short-chain fatty acids and lactate and intestinal pH were determined. Duration of feeding did not influence the outcome of any of these measurements and, thus, data were pooled to test effects of "basal diet" and "dietary inulin content" on selected parameters. Intestinal microbiota composition in the experimental pigs was highly individual. This phenomenon made it difficult to assess the influence of dietary intervention on bacterial communities in the gut and no clear-cut effects of treatment were observed. Since inulin is claimed to increase intestinal numbers of lactobacilli and bifidobacteria, these two bacterial groups were of special interest. However, lactobacilli were not influenced by inulin feeding. Bifidobacteria were detected in 40 % of the animals when inulin was present in the diet. In contrast, only 13 % of the pigs harbored bifidobacteria when inulin-free diets were fed. These effects were independent of the type of basal diet. Intestinal SCFA production was higher and pH was lower when the cereal-based diets were fed. When inulin was added, the relative contribution of butyrate to the total SCFA was increased. In conclusion, this study did not support a strong prebiotic effect of inulin in pigs. Intestinal microbiota of the experimental pigs was individually composed and did not respond to dietary intervention. The dietary background of naturally occurring non-digestible carbohydrates was not an important factor masking possible prebiotic effects of inulin.

The second well-established concept of microbiota modulation is the dietary application of probiotic bacteria. In animal nutrition, *Enterococcus faecium* NCIMB 10415 belongs to the probiotic strains with a long history of safe application and its use is authorized in the European Union. To test whether or not the strain is active against gut inflammation, an experiment with IL-10$^{-/-}$ mice was conducted (PUBLICATION 10). After weaning, one group of mice was fed a diet containing ~$\log_{10}$ 6.5 cells of NCIMB 10415 per g. The control animals were fed the same diet without the strain. Animals were killed after 3, 8, and 24 weeks of intervention, respectively. All mice developed mild colitis and no clear-cut differences between the treated and untreated animals were observed. This was also true for the relative expression at the mRNA level of TNFα, IL-6, and IL-23 in the intestinal mucosa. The measurement of paracellular permeability and of epithelial and subepithelial ion conductance did not indicate that NCIMB 10415 improved epithelial barrier functions. In addition, the treated and untreated mice did not differ in intestinal microbiota composition as concluded from similar band patterns in denaturing gradient gel electrophoresis and from 16S rRNA gene sequencing analyses. In summary, *E. faecium* NCIMB 10415 does not influence the onset and perpetuation of chronic gut inflammation in the IL-10$^{-/-}$ mouse model.

General Discussion

Intestinal bacteria play an important role in host health and disease but the underlying mechanisms have so far not been fully unraveled. There is strong evidence that diet is one of the most important factors that influence intestinal microbiota composition and function. Therefore, it can be hypothesized that bacterial effects on host physiology can be modulated by dietary intervention. However, it is difficult to study interactions between gut bacteria, the host and diet because the composition of the intestinal microbiota is highly complex and individually variable. To reduce the number of confounders that influence gut microbiota composition, experiments can be conducted under highly controlled conditions in inbred rodent models. Inbred rodents provide the advantage that they share the same genetic background. It is therefore very likely that significant inter-individual differences in host physiology or immunology, which may influence gut microbiota, are reduced in these experimental animals. In addition, studies in gnotobiotic animals can be conducted much more easily in rodents than for instance in pigs. Gnotobiotic animals per definition provide the advantage that the intestinal microbiota composition is known and that bacterial functions of interest can be studied in detail. Using such rodent models but also experimental pigs, (i) the influence of host genetics and health on intestinal microbiota composition, (ii) the responses of intestinal bacteria towards changes in dietary composition, (iii) the relevance of bacterial modification of dietary components for their availability and/or biological function and (iv) the efficacy of intestinal microbiota modulation by the use of pre- and probiotics were addressed. In the following, the relevant findings of the respective studies are discussed in a more general context.

Host genes influencing microbiota composition remain to be identified

It has been proposed that genetic host factors are involved in the selection of an individual set of bacterial species in the gut. Many of the genes that have been implicated in microbiota regulation play a role in the recognition by the innate immune system of bacterial antigens. For instance, nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-deficient and wild-type control mice differ in gut microbiota composition (Petnicki-Ocwieja *et al.*, 2009; Rehman *et al.*, 2011). In addition to NOD2, Toll-like receptor- (TLR-) dependent mechanisms may regulate microbiota composition because many TLRs are expressed in the intestine and sense highly conserved bacterial molecules (Wells *et al.*, 2011). Bacterial antigen recognition by TLRs initiates the “defence program” of Paneth cells (Vaishnava *et al.*, 2008) and this activation results in the production of antimicrobial peptides which are

important regulators of microbiota composition (Meyer-Hoffert *et al.*, 2008; Salzman *et al.*, 2010).

To test whether the recognition by the immune system of bacterial cell wall constituents by TLR2 and TLR4 influences gut microbiota composition, an experiment with germ-free TLR2/4 double knock-out and the respective wild-type C57BL/10 mice was conducted (PUBLICATION 1). The microbiota that developed in these animals after inoculation with the same bacterial cocktail was very similar and there was no effect of TLR2 and TLR4 on gut bacteria. In contrast, microbiota composition in identically treated C3H and BALB/c mice different from that in all mice with a C57BL/10 background. The observation that inbred mouse strains differ in intestinal microbiota composition is in line with findings by others (Friswell *et al.*, 2010; Kovacs *et al.*, 2011) and indicative of a host-specific control of bacterial gut colonization. As mentioned above, microbiota transfer experiments did not support the hypothesis that TLR2 and TLR 4 belong to the host factors that contribute to this phenomenon. In contrast, the importance of bacteria-TLR interactions for bacterial gut colonization has been demonstrated by others and the underlying molecular mechanisms have been unraveled in mice monoassociated with a wild-type *Bacteroides fragilis* strain or with a *B. fragilis* mutant defective of capsular polysaccharide A (PSA) production. The wild-type strain was capable of colonizing the gut wall of mice but the mutant lost this ability. In a series of *in vivo* and *in vitro* experiments it was clearly worked out that the *B. fragilis* PSA induces tolerance through TLR2 signaling. This feature enables *B. fragilis* to colonize the gut in close proximity to the intestinal epithelium (Round *et al.*, 2011). In conclusion, the exact role of TLR2 and TLR4 in controlling microbiota composition needs further clarification. The same is true for a possible role of host genes other than *TLR2* or *TLR4*.

In order to identify genes that govern microbiota composition, a study in C3H and C57BL/10 mice was performed (Brodziak *et al*., 2013). The two mouse strains were selected since they displayed in a previous experiment the highest inter-strain differences in intestinal microbiota composition. The experiment was essentially performed as described in CHAPTER 1 and supported the notion that the host genotype at least in part influences gut microbiota composition. In this study, messenger RNA was extracted from the intestinal mucosa of the mice and subsequently subjected to global gene expression analysis with a mouse genome micro-array chip. Genes with the highest expression differences encoded antimicrobial factors and receptors for bacterial antigens. Several interferon-inducible genes were more highly expressed in C3H than in C57BL/10 mice. In addition, a frameshift mutation was detected in C57BL/10 mice resulting in a defective gene encoding the antibacterial phospholipase A2, group IIA. This finding was in line with an approximately 70-fold higher expression of this gene in C3H mice. Taken together, this study indicated that genes involved in immune functions play an important role in the development of individual bacterial

communities in the intestine. However, a number of open questions have to be addressed in future studies. For instance, it is not clear if differences in mucosal gene expression are cause or consequence of differences in microbiota composition. In addition, it is unclear if the results obtained in different inbred mouse strains are transferable to other mammalian species.

It has been mentioned in CHAPTER 1 that, in addition to the host genotype, environmental factors contribute to the development of host-specific bacterial communities in the gut. In the respective experiment, C57BL/10, BALB/c and C3H mice were housed in mixed groups in two different cages. The comparison of microbiota composition in these mice revealed that even under highly controlled experimental conditions so far unspecified factors influence microbiota composition. This observation is in line with findings by others (Hufeldt *et al.*, 2010; Fukuda *et al.*, 2011) and in one study the microbiota was even more affected by environmental than by genetic factors (Friswell *et al.*, 2010). It has been proposed that a triangular relationship between polygenic host traits, intestinal bacteria and environmental factors governs microbiota composition and function (Benson *et al.*, 2010) and there is strong evidence that nutrition plays a particular role in this context. Owing to the assumed importance of nutrition, its influence on microbiota composition and function has been investigated in more detail. The most important results of these studies are discussed below (see page 33 ff.).

Intestinal microbiota composition is affected by host health

In addition to host genetics, chronic gut inflammation has been considered an important factor that affects intestinal microbiota composition (Frank *et al.*, 2011). To characterize responses of gut bacteria towards chronic colitis, experiments in mice were conducted. In the first experiment, intestinal microbiota composition in colitic IL-10$^{-/-}$ and healthy wild-type mice was compared (PUBLICATION 2). Chronic gut inflammation in the IL-10$^{-/-}$ mouse model was associated with a reduced microbiota diversity in conjunction with a bloom of *E. coli*. This finding was expected since low intestinal microbiota diversity and an increased abundance of enterobacteria is frequently observed in gut inflammation (Kaser *et al.*, 2010). The high numbers of one single *E. coli* O7:K1:H7 strain in IL-10$^{-/-}$ mice offered the opportunity to investigate specific features of this strain with possible implications in host health. Some of the strain's traits have been reported previously in colitis-associated *E. coli*. For instance, *E. coli* strains belonging to the virulence-associated phylogenetic group B2 are more frequently detected in the inflamed gut than other phylotypes (Petersen *et al.*, 2009). In addition, genes that were detected in the O7:K1:H7 strain's genome such as serine protease autotransporters have been implicated in the development of gut inflammation (Kotlowski *et*

al., 2006). Moreover, *E. coli* adherence to and invasion of host cells is a critical feature of *E. coli* in the inflamed intestine (Barnich and Darfeuille-Michaud, 2007) and the ability to internalize iron increases bacterial pathogenicity (Perl *et al.*, 2004). However, additional in vitro and in vivo experiments provided no evidence for pro-inflammatory properties of the isolated O7:K1:H7 strain. Therefore, it was concluded that a high number of fitness- and virulence-associated genes enables commensal bacteria to cope with hostile environmental conditions in the inflamed gut. The observation that the *E. coli* isolate was able to outcompete other *E. coli* strains from the healthy gut of gnotobiotic mice indicated that these traits not only provide a selection advantage under inflammatory but also under physiologic conditions. The notion that virulence-associated *E. coli* strains are generally well-equipped for the survival in the gut is supported by the observation that B2 *E. coli* strains are often dominant enterobacteria in healthy human subjects (Zhang *et al.*, 2002; Nowrouzian *et al.*, 2005) and in pigs (Schierack *et al.*, 2008). In summary, gut inflammation is associated with profound changes in microbiota composition. Whether reduced microbiota diversity and a bloom of *E. coli* in the IL-10$^{-/-}$ mouse are cause or consequence of gut inflammation remains elusive.

Highly adapted bacterial species may benefit from changes in the intestinal milieu in the inflamed colon. To identify possible inflammation-associated environmental changes, a second experiment in IL-10$^{-/-}$ and healthy control mice was conducted (PUBLICATION 3). In these mice, concentrations of cholesterol and coprostanol and of primary and secondary bile acids were measured. In addition, microbiota composition was addressed. Gut inflammation in IL-10$^{-/-}$ mice was associated with an impaired intestinal metabolism of neutral sterol and bile acids which is in line with previous observations in humans (Kruis *et al.*, 1986; Nyhlin *et al.*, 1994; Hrabovsky *et al.*, 2009). It was not possible to clarify whether the observed changes in intestinal neutral steroid concentrations caused or resulted from inflammation-associated changes in gut microbiota composition. Both possibilities are supported by literature reports. On the one hand microbiota composition and function are modified by dietary cholesterol supplementation (Tamura *et al.*, 2009). On the other hand, the degree of intestinal cholesterol transformation strongly depends on bacterial community structure in the gut (Veiga *et al.*, 2005). The relevance of bacterial transformation for intestinal bile acid profiles is more clear since primary bile acids are exclusively converted by bacterial enzymes to secondary bile acids (Ridlon *et al.*, 2006). The secondary bile acids are known to preferentially inhibit the growth of anaerobic bacteria (Floch *et al.*, 1972) and may thereby change microbiota composition. The reduced diversity in conjunction with the dominance of only few bile acid-resistant bacteria in IL-10$^{-/-}$ mice suggested that this was the case in the inflamed gut.

Taken together, the results obtained in PUBLICATION 2 and PUBLICATION 3 supported the hypothesis that hostile conditions in the inflamed gut exert a selection pressure on intestinal bacteria. In this way, the significant differences between healthy and colitic mice in microbiota composition may rather be result than cause of the disease.

Diet influences intestinal microbiota composition

Nutrition is considered an important factor that influences intestinal microbiota composition and function. Therefore, specific responses of gut bacteria towards dietary intervention have been investigated in more detail. In a first experiment, effects of two different high-fat diets on gut microbiota composition were studied in mice (PUBLICATION 4). In this study, germ-free mice were used to explore if the presence or absence of gut bacteria influences body mass and body fat gain when different energy-rich diets are fed. The high-fat diets were nearly identical in their crude fat, crude carbohydrate and crude protein content. However, one of the diets (a commercially available Western-style diet, WD, which is widely used in obesity research) was rich in beef tallow and hydrogenated vegetable oil ("vegetable shortening") with high concentrations of trans-fatty acids. Highly digestible sucrose and maltodextrin contributed significantly to total carbohydrates. The second diet (HFD) contained different plant oils and starch as fat and carbohydrate source, respectively. Both high-fat diets and the low-fat control diet (LFD) were poor in fermentable fiber. It has previously been reported that germ-free mice on WD do not gain weight and that germ-free mice are generally protected from diet-induced obesity (Backhed *et al.*, 2007). In contradiction to this proposition, germ-free mice became more obese on the HFD than their conventional counterparts. Interestingly, reduced body mass and body fat gain in germ-free mice was reproducible when WD was fed, indicating that the specific nature of dietary ingredients has to be considered in research on the role of bacteria in diet-induced obesity.

To study specific responses of gut bacteria towards the different types of experimental diets, a fluorescence in situ hybridization (FISH) approach was applied. The switch from chow to HFD and WD at the beginning of the experiment was associated with a decrease of bacteria belonging to the *Bacteroides-Prevotella* group (Bacteroidetes) and the *Eubacterium rectale-Clostridium coccoides* cluster, the *Clostridium leptum* group and the *Enterococcus-Lactobacillus* group (Firmicutes). This effect was more pronounced when the WD was fed. It was concluded that dominant bacteria in the murine gut decrease at the expense of microbes that are not detectable by the selected probes in FISH analyses. Sequence analysis of bacterial 16S rRNA genes revealed that an increased abundance of Erysipelotrichaceae was the most significant effect of high-fat feeding. This group within the Firmicutes is not detectable with the applied probe set and, therefore, the increased abundance of

Erysipelotrichaceae was considered a valid explanation for the observed differences between the study groups. The strong response of Erysipelotrichaceae towards high-fat diets is in line with previous observations (Turnbaugh *et al.*, 2008). In this study, an increase of Mollicutes in mice fed the WD was demonstrated with a deep-sequencing approach. The re-evaluation of the published 16S rRNA gene sequences revealed that the majority of these Mollicutes sequences belong to the Erysipelotrichaceae.

The experiments with germ-free and conventional mice and different types of diet supported the hypothesis that interactions between gut bacteria, host and dietary ingredients play a role in obesity development but the exact nature of these interactions and the specific contribution of bacteria, diet and host remains to be elucidated. There is some indication that bacterial communities in the gut are influenced by the host phenotype. For instance, intestinal microbiota composition in leptin-deficient hyperphageous mice with genetically-driven obesity is very similar to that in mice with diet-induced obesity (Ley *et al.*, 2005; Turnbaugh *et al.*, 2008). However, it has not become entirely clear if gut bacteria respond preferentially to diet or the host phenotype. In one experiment mice have been switched back to normal chow after 12 weeks of high-fat diet feeding. Feeding the high-fat diet induced significant alterations in microbiota composition However, bacterial species richness and microbiota similarity became more similar to that in chow-fed mice after 4 weeks and complete convergence was observed after 10 weeks. This phenomenon was accompanied by a significant reduction in body mass (Zhang *et al.*, 2012). Effects of extreme differences in nutrition and nutrient availability on microbiota composition have been studied in children. Italian children consuming a normal Western-style diet, which is rich in highly digestible sugars and fat, show the expected microbiota composition at the phylum level. The microbiota in these children is dominated by Firmicutes (51 % of total bacterial 16S rRNA gene sequences) followed by Bacteroidetes (27 %) and others (22 %). In contrast, microbiota in children from rural Burkina Faso consuming a diet essentially composed of millet and sorghum is dominated by Bacteroidetes (73 %) and Firmicutes make only up to 12 % of total bacteria (De Filippo *et al.*, 2010). Although the African children did not suffer from malnutrition, it can be assumed that the extreme differences in nutrient availability significantly influence the children's body fat and lean mass. It is therefore unclear if the dominance of Firmicutes in European children and of Bacteroidetes in African children resulted from different nutrition habits or the resulting physical condition or body composition. Strong evidence for a more important role of diet for microbiota composition and function comes from a study in adult human subjects. In this study, dietary intervention with high-fat diets resulted in microbiota changes within 24 hours. Long-term dietary habits were associated with the formation of enterotypes according to the dominant genera in the intestine (Wu *et al.*, 2011). Rapid responses to dietary intervention have also been reported

for experimental animals: changes in microbiota composition in mice on a high-fat diet are an early event that can be observed before the mice become obese (Patrone *et al.*, 2012). Moreover, adaptation at the gene expression level and differences in the statistical representation of metabolic pathways in the microbiome of mice can be observed after feeding high-fat diets for only one single day (Turnbaugh *et al.*, 2009a). The most striking evidence for the particular role of diet has been provided by a study in mice that are genetically protected from diet-induced obesity. After feeding a high-fat diet for 21 weeks, microbiota composition in these mice had shifted into the same direction as in identically treated wild-type mice with an obese phenotype (Hildebrandt *et al.*, 2009). Interestingly, nutrition influences gut microbiota composition more strongly than the genetic background and even the phylogenetic position of the host organism. Bacterial community structure in humans, zoo and free-living animals clustered more significantly according to their carnivorous, omnivorous or herbivorous nutrition than phylogeny (Ley *et al.*, 2008). A later study confirmed these results and demonstrated by deep sequencing that the same is true for bacterial functions that are enriched in the respective microbiomes (Muegge *et al.*, 2011). Taken together, a number of studies strongly support the notion that gut bacteria respond more strongly to nutrition than to the physiological status of the host. If this assumption can be further substantiated, the dietary ingredients exerting these effects need to be identified.

As mentioned above, the most significant changes in fecal microbiota composition are observed when mice are switched from a standard rodent chow to purified experimental diets. This bacterial response is not surprising since rodent chow is rich in complex fiber material and other compounds that serve as substrates for intestinal bacteria. In contrast, purified diets in nutrition research usually consist of highly digestible ingredients and poorly fermentable cellulose. It can be assumed that only a given proportion of gut bacteria is capable of using the remaining diet- and host-derived substrates and that these bacteria have a selection advantage under substrate-limiting conditions. The different effects of the two high-fat diets reported in PUBLICATION 4 are more difficult to explain. Both diets were composed of highly digestible and poorly fermentable ingredients. Therefore, similar effects on microbiota composition were expected but the observed bloom of Erysipelotrichaceae was more pronounced in mice on HFD. Unfortunately, it was not possible to clarify which ingredients are responsible for these effects. Since HFD and WD differed in their fat sources, it can be hypothesized that the nature of dietary fat is an important factor. This assumption is supported by the finding that the proportion of intestinal Bacteroidetes decreases by approximately 30 % when mice consume a diet rich in saturated fats. Feeding diets rich in polyunsaturated fatty acids decreases the proportion of Bacteroidetes by approximately 10 % (Liu *et al.*, 2012). Whether or not members of the Erysipelotrichaceae respond to differences in dietary fat in a similar way should be investigated in future experiments. The use of

gnotobiotic animal models and defined diets in such experiments may help to specifically identify interactions between gut bacteria and dietary ingredients.

It is difficult to study specific effects of selected dietary components and to unravel the underlying mechanisms in conventional animals. Therefore, a gnotobiotic animal model for studying host-microbe and diet-microbe interactions has been established. This model was designed to fulfill the following criteria: (i) the selected bacteria should represent numerically dominant organisms in the gut, (ii) the metabolic activities of this model community should mimic that of a complex microbiota, (iii) the genome sequence of all community members should be available, (iv) the model consortium should form a stable community in rodents and should be transferable from one to the next generation, and (v) the composition of the model community should be modifiable (Wohlgemuth *et al.*, 2010). To meet these requirements, a community consisting of *Anaerostipes caccae*, *Bacteroides thetaiotaomicron*, *Bifidobacterium longum*, *Blautia producta*, *Clostridium butyricum*, *Clostridium ramosum*, *Escherichia coli* and *Lactobacillus plantarum* was successfully established in previously germ-free rats (PUBLICATION 5). This model (referred to as SIHUMI) was subsequently used to test specific influences of selected dietary ingredients on microbiota composition. In previous experiments, profound changes in microbiota composition have been observed when conventional mice were switched form a standard rodent chow to purified diets. Therefore, SIHUMI rats were fed in a first experiment either chow or a semi-synthetic diet low in fermentable fiber. The latter diet was later used as low-fat control diet (LFD) when WD was fed to SIHUMI rats. Compared to standard chow, LFD feeding resulted in a significant decrease in total bacterial numbers. The strongest decrease in cell numbers was observed for *Bifidobacterium longum* and *Blautia producta* indicating that these two species are more susceptible to substrate depletion than the other consortium members. It was subsequently tested whether the model consortium responds to the addition of pectin and inulin, respectively. The two indigestible but fermentable substrates were selected because they have been shown to preferentially promote the growth of bifidobacteria (Kruse *et al.*, 1999; Shinohara *et al.*, 2010) and may function as a so-called prebiotic. However, a bifidogenic effect was neither observed for pectin nor inulin. Therefore, the model may require further modifications for the study of potential prebiotic effects of selected dietary supplements. In addition, the response of the model community to WD was tested. It turned out that numbers of *C. ramosum*, a member of the Erysipelotrichaceae, were significantly higher in rats on WD than in rats on LFD. This finding supported the notion that Mollicutes (Turnbaugh *et al.*, 2008) and, more specifically, Erysipelotrichaceae (PUBLICATION 4) play a role in diet-induced obesity. Taken together, it has been shown that the simplified intestinal microbiota consisting of *A. caccae*, *B. thetaiotaomicron*, *B. longum*, *B.*

producta, *C. butyricum*, *C. ramosum*, *E. coli* and *L. plantarum* is a useful model for studying dietary influences on bacterial functions with relevance to host physiology.

To develop the SIHUMI model further, mice colonized by a complete SIUMI and by a SIUMI that does not include *C. ramosum* (SIHUMI-*Cra*), respectively, are currently used. In preliminary experiments, SIHUMI-*Cra* mice did not differ in weight gain when fed either high- or low-fat diets indicating that the absence of *C. ramosum* protects from diet-induced obesity. In contrast, control mice with a complete SIHUMI behaved as expected and became obese on the high-fat diet. Most importantly, it is possible to reproduce the adipogenic effect of the complete SIHUMI in mice that are exclusively colonized with *C. ramosum* (Woting A, Loh G, Blaut M: Role of *Clostridium ramosum* (Erysipelotrichaceae) in obesity development. Oral presentation at the 5th Seeon conference Microbiota, Probiota and Host; June 15, 2012.). In future studies, differences between the SIHUMI and the SIHUMI-*Cra* in bacterial metabolite production and protein expression will be addressed in order to identify adipogenic factors of *C. ramosum*.

Gut bacteria respond at the cellular level to changes in dietary composition

The use of animal models with reduced microbiota complexity offers the advantage that bacterial responses towards diet can be studied at the molecular level. In a first experiment that aimed to identify such responses, mice were monoassociated with the *E. coli* laboratory strain MG1655 (PUBLICATION 6). These mice were fed diets that were composed in such a way to influence selected bacterial metabolism pathways. For instance, a lactose-rich diet was used to induce the expression of enzymes involved in *E. coli* carbohydrate metabolism. Since this diet significantly affected the expression of enzymes of the Leloir pathway (galactose utilization), the experimental approach was considered appropriate to study bacteria-diet interactions. An unexpected finding was that lactose feeding resulted in an up-regulation of proteins with protective functions against oxidative stress. This study provided novel information on *E. coli* physiology. In addition, the experiment clearly demonstrated that responses towards nutrition can be identified at the protein expression level. It should be considered that the experimental approach presented here suffers from the following limitations. *E. coli* is not a dominant member of the intestinal microbiota. In addition, the laboratory strain MG1655 may have lost typical features of a gut bacterium. Limitations of the animal model include the fact that monoassociated animals do not reflect the physiological status of conventional animals. Bacteria-bacteria interactions are per definitionem excluded under these conditions. However, it may be possible in future studies to extend this approach to identify responses of more dominant bacteria towards more relevant dietary interventions. In such experiments, hypotheses may be generated by the application of metagenomic

techniques to complex bacterial communities. For instance, it has been demonstrated by deep sequencing that the intestinal metagenome in mice on high-fat diets is enriched in genes of bacterial fructose and mannose metabolism, glycolysis/gluconeogenesis and carbon fixation. In contrast, genes implicated in pentose and glucuronate interconversion or starch and sucrose metabolism are depleted under these conditions (Turnbaugh *et al.*, 2008). The biological relevance of changes in these functions for bacterial metabolism and host physiology may be tested in reductionist models taking advantage of gnotobiotic animals.

Gut bacteria modulate availability and biological function of dietary ingredients

The breakdown of undigested dietary compounds and the production of SCFA are important functions of gut bacteria (Macfarlane and Macfarlane, 2011). In addition, intestinal bacteria interact with minerals and trace elements and with non-nutritive plant compounds. Very little is known about effects of gut bacteria on the bioavailability of trace elements. However, there is some indication that gut bacteria compete with the host for dietary selenium (PUBLICATION 7). In this study, selenium bioavailability was lower in conventional than in germ-free mice. Presence of gut bacteria was associated with reduced expression levels and activities of selenium-containing enzymes in liver, plasma and intestinal mucosa. Selenoproteins have been implicated in a number of important physiological processes. They have enzymatic redox functions, play a role in peroxide removal, repair of oxidized proteins and cell membrane compounds and in the regulation of redox signaling. In addition, they are involved in thyroid hormone metabolism, selenium storage and transport and protein folding (Papp *et al.*, 2007). Taken together, selenium is an essential trace element and adequate dietary selenium supply is important for many biological functions. When basal selenium supply is poor, competition between gut bacteria and their host may influence selenium availability and host physiology. However, it is not very likely that bacterial selenium sequestration is of importance in humans with well-balanced nutrition habits and in animal nutrition with well-supplemented diets.

In addition to macro- and micronutrients, dietary plant material contains secondary plant metabolites including lignans. Important dietary sources for lignans are whole grain cereals, vegetables and fruits (Adlercreutz, 2007). Upon digestion, lignans undergo bacterial transformation in the intestine. This transformation influences bioavailability and bioactivity of these substances. The principal steps of lignan transformation by intestinal bacteria have been well characterized under in vitro conditions: a bacterial consortium consisting of the four human gut-derived species *Clostridium saccharogumia*, *Blautia producta*, *Eggerthella lenta* and *Lactonifactor longoviformis* is capable of converting secoisolariciresinol diglucoside

(SDG) into the enterolignans enterodiol (ED) and enterolactone (EL) (Blaut and Clavel, 2007). As mentioned above, transformation by gut bacteria of lignans is an important factor that influences their bioactivity. Upon activation, lignans may exert protective effects against cardiovascular and metabolic diseases. In addition, they are claimed to protect from hormone-dependent cancers (Adolphe *et al.*, 2010). Although the anti-cancer effects of lignans have extensively been investigated, epidemiological studies do not provide a clear picture. Some studies suggest that a high intake of dietary lignans is protective against breast cancer but other studies do not support this assumption. Methodological differences (Sonestedt and Wirfalt, 2010) and differences in the abundance of lignan-transforming bacteria in study populations (Clavel *et al.*, 2006) have been proposed to contribute to this conflicting study outcomes.

The observation that chemically induced breast tumor burden is lower in rats exclusively colonized with lignan-converting bacteria (LCC) than in germfree rats on a lignan-rich diet highlights the importance of bacterial enterolignan production for the anti-cancer effects of lignans (PUBLICATION 8). The reduced tumor burden in LCC rats resulted from a lower mitotic activity of tumor cells in conjunction with a higher apoptotic rate. The experiment did not support the notion that the beneficial effects of lignans were brought about by estrogen-dependent pathways. In contrast, bacterial lignan transformation increased the activities of oxidant-degrading enzymes in LCC rats indicating a reduced susceptibility towards oxidative stress in these animals. However, similar concentrations of oxidative stress markers in all animals did not support this assumption. Taken together, the bacterial transformation of the plant lignan SDG to the enterolignans ED and EL is the prerequisite for the anti-cancer effects of lignans in this model but the underlying mechanisms require further investigation. These investigations should focus on the molecular effects of ED and EL on cellular pathways influencing cell proliferation and apoptosis.

To generate new hypotheses that can be addressed in future studies, protein was extracted from mammary tumor tissue obtained from germ-free and LCC rats. When this protein was subjected to proteome analysis, the induction of 13 proteins and the repression of 11 proteins were observed in LCC rats. Among these proteins, ranBP-type and C3H4-type zinc finger-containing protein 1 (RBCK1) and poly(rC)-binding protein 1 (PCBP1, also referred to as αCP1) displayed lower expression in LCC than in germ-free rats (PhD thesis Hoda Mabrok, University of Potsdam, Institute of Nutritional Science, 2013). Interestingly, RBCK1 and PCBP1 depletion is associated with cell cycle arrest and decreased cell proliferation (Waggoner *et al.*, 2009; Gustafsson *et al.*, 2010). It is now possible to test whether or not enterolignans are capable of influencing RBCK1 and PCBP1 expression and, if yes, this effect is involved in the reduced cell proliferation and increased apoptotic rate that have been observed in tumors from LCC rats.

Modulation of gut microbiota composition by pre- and probiotics is not always effective

Intestinal bacteria influence host physiology and health. This observation has triggered the development of strategies for the modification of intestinal microbiota composition and function. For instance, in-feed antibiotics have been used for decades. The use of these feed additives has increased productivity of farm animals and it is likely that these effects have been brought about by intestinal microbiota modulation (Dibner and Richards, 2005). The use of antibiotic growth promoters in the European Union is no longer permitted since 2006 and their ban has put forward the search for alternative feed additives. Well-established concepts for targeted microbiota modification include the use of probiotics and prebiotics. The definition of the latter includes a wide range of dietary compounds but the majority of prebiotic feed and food additives are non-digestible oligosaccharides. According to the definition of prebiotics, their application should confer health benefits to the host organism (Gibson *et al.*, 2010). The prebiotic-associated benefits in human nutrition include reduced gastrointestinal infections or colon cancer prevention (Roberfroid *et al.*, 2010). More relevant in animal nutrition are effects of prebiotics on performance-associated parameters. However, reported effects of inulin-type prebiotics on animal performance and nutrient digestion vary and specific modulation of intestinal microbiota is not always observed (Verdonk *et al.*, 2005).

The use of different types of fiber may be responsible for inconsistent effects of prebiotics in animal experiments. For instance, an increase of intestinal bifidobacteria and lactobacilli in pigs has been reported in response to galactooligosaccharide feeding (Smiricky-Tjardes *et al.*, 2003). The bifidogenic effect of these oligosaccharides in pigs has been confirmed by others. In this study, inulin also fulfilled the criteria of a prebiotic feed additive (Tzortzis *et al.*, 2005). Others failed to demonstrate prebiotic effects of both lactulose and inulin (Branner *et al.*, 2004) or fructooligosaccharide-rich Jerusalem artichoke flour (Farnworth *et al.*, 1992). The composition of the basal experimental diet which is used to test effects of different types of fiber may be an additional factor that influences the outcome of studies on prebiotics. For instance, pig diets mainly consist of cereal grain which contains considerable amounts of short-chain fructooligosaccharides (van Loo *et al.*, 1995). These naturally occurring fructans may mask the effects of potential prebiotics. However, no differences in pig performance were observed when inulin was fed together with cereal-based and semi-synthetic diets, respectively (PUBLICATION 9). Expected effects of inulin on gut microbiota composition included increased numbers of bifidobacteria and/or lactobacilli in the experimental pigs. This assumption was based on the observation that many gut bacteria are capable of fructan degradation but mainly bifidobacteria are stimulated at the expense of potential pathogens (Gibson *et al.*, 2010). However, no clear-cut inulin effect on microbiota composition was

observed. It was concluded that the intestinal microbiota in pigs is already highly adapted to fiber-rich diets and, thus, does not respond to prebiotic treatment.

Alternatively, the amount of inulin that entered the large intestine of the experimental pigs may have been too low to exert significant effects on intestinal microbiota composition. Inulin is thought to pass through the small intestine without degradation and to reach the large intestine intact. In fact, the cecum has been reported to be the major degradation site when inulin is fed to pigs (Yasuda *et al.*, 2007). In contrast to this study, inulin was almost completely degraded in the small intestine when cereal-based and semi-synthetic diets were fed to pigs and less than 0.2 % of the ingested inulin was recovered from the large intestine. The hypothesis that this amount of inulin was too low to induce changes in microbiota composition is supported by findings in pigs experimentally infected with *Brachyspira hyodysenterica*. In these animals, dietary inulin at concentrations of 8 % but not 2 % and 4 % reduced the incidence of swine dysentery. The authors concluded from elevated SCFA concentrations in the pigs fed the highest concentration of inulin that this effect was brought about by changes in gut microbiota composition (Hansen *et al.*, 2011). High hygienic standard in experimental animal facilities may be an additional factor that influences effectiveness of prebiotics in pig studies. This notion is supported by different effects of the same inulin preparation on microbiota composition in pigs reared under well-controlled experimental conditions or at a commercial farm (Janczyk *et al.*, 2010). Taken together, different types of prebiotics may differ in their effects in pigs. High amounts of prebiotic supplements may be necessary to influence the porcine microbiota composition and function.

In addition to prebiotics, probiotic bacteria are used to modulate intestinal microbiota. Per definition, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (http://www.who.int/foodsafety/publications/fs_management/probiotics2/en). It has been proposed that probiotic effects are mediated by modulation of intestinal microbiota composition, by direct effects on host physiology or a mixture of both. Probiotic-induced changes in microbiota composition are possibly brought about by competitive exclusion mechanisms. For instance, probiotics may compete with pathogenic bacteria for epithelial binding sites such as glycoconjugate receptors at the epithelial surfaces. Other mechanisms involved in microbiota modification by probiotics include the production of antibacterial substances. Direct interactions between probiotic bacteria and the host include the modulation of host immune functions through stimulation of TLR-activated pathways. In addition to immunomodulatory effects, probiotics can also alter the barrier function of the intestinal epithelium and its integrity. The latter may result from probiotic effects on tight junction complexes (Wohlgemuth *et al.*, 2010). These mechanisms may be involved in beneficial effects of *Enterococcus faecium* NCIMB 10415 in pigs. This strain has been shown in piglets to improve daily weight gain in conjunction with reduced

severity of diarrhea (Zeyner and Boldt, 2006). The latter effect has also been observed by others but in these studies NCIMB 10415 failed to improve performance-associated parameters (Broom *et al.*, 2006; Taras *et al.*, 2006). Thus, it can be hypothesized that anti-inflammatory effects belong to the most important features of the strain. However, it is unclear how such anti-inflammatory effects are brought about since NCIMB 10415 is not capable of influencing intestinal microbiota composition (Broom *et al.*, 2006) or reducing pathogenic *Escherichia coli* strains in piglets (Taras *et al.*, 2006). Moreover, cell numbers in feces and internal organs of *Salmonella enterica* Typhimurium increase in response to NCIMB 10415 treatment when pigs are experimentally infected with this pathogen (Szabo *et al.*, 2009). This rather adverse effect may result from reduced immune responses towards intestinal pathogens which can be deduced from lower levels of fecal immunoglobulin (Ig) A, circulating IgG and epithelial cytotoxic CD8+ T-cells when NCIMB 10415 is fed to piglets (Scharek *et al.*, 2005; Scharek *et al.*, 2007).

To investigate the mode of probiotic action of NCIMB 10415 under highly controlled experimental conditions, an experiment in IL-$10^{-/-}$ mice was conducted (PUBLICATION 10). NCIMB 10415 was not effective against gut inflammation in this experiment and did not influence intestinal microbiota composition, the barrier function of the intestinal epithelium or the expression of selected genes encoding pro-inflammatory cytokines. An interesting finding in this study was a reduced occurrence of *Akkermansia muciniphila* in mice that were treated with NCIMB 10415 for eight weeks. This finding was associated with a reduction of cecal inflammation severity, lower paracellular permeability of the intestinal epithelium and a lower expression of interferon-gamma and interferon-gamma-induced protein 10 than in all other NCIMB-treated mice. *A. muciniphila* is a mucin-degrading bacterium which has been isolated from the human gut (Derrien *et al.*, 2004). Its role in gut health has not been fully understood but its numbers are reduced in chronic gut inflammation (Png *et al.*, 2010). Monocolonization of mice with *A. muciniphila* induces molecular pathways involved in epithelial cell membrane modifications, sulfur metabolism and immune cell maturation (Derrien *et al.*, 2011). Whether or not interactions between *A. muciniphila*, NCIMB 10415 and the host influence gut health remains elusive. In summary, the targeted modulation of intestinal microbiota composition and function has the potential to increase the well-being and the productivity or performance of the host organism. However, effects of pre- and probiotics are sometimes difficult to evaluate.

Synopsis and Final Conclusion

The most important findings of the studies presented in this thesis are:

1) The recognition by the immune system via the Toll-like receptors TLR2 and TLR4 of bacterial antigens is not involved in the development of a host-specific intestinal microbiota. The nature of genetic host factors that play a role in the establishment an individual bacterial community in the intestine of a given host remains to be elucidated. A combination of holistic and hypothesis-driven approaches may help to identify the respective genes. Environmental factors including host health and nutrition may exert stronger effects on gut bacteria than the host genotype.

2) Reduced gut microbiota diversity in conjunction with an increased abundance of few bacterial species, in particular of *E. coli*, is a critical feature of chronic colitis. It is still not clear whether changes in microbiota composition are one of the causes or a consequence of the disease. However, there is evidence that altered environmental conditions in the inflamed colon influence the structure of bacterial communities. The respective changes may in turn aggravate intestinal inflammation. Since chronic colitis is associated with impaired intestinal neutral steroid and bile acid metabolism, the modification of intestinal steroid profiles by pharmaceutical or dietary intervention may help to stabilize a “healthy microbiota”.

3) Members of the Erysipelotrichaceae increase in the intestine of mice on high-fat diets. There is evidence that members of this family strongly influence the severity of diet-induced obesity. Therefore, their specific role in the development of an obese phenotype and the associated metabolic disorders should be investigated in more detail. Studies on this specific role may benefit from the use of gnotobiotic animal models with a simplified intestinal microbiota. In such models bacterial responses at the cellular level towards dietary intervention can be studied.

4) The intestinal microbiota competes with the host for nutrients. Bacterial scavenging of dietary selenium results in a reduced expression and activity of host selenoproteins. Owing to the importance of these proteins in various cellular processes, this reduction may significantly influence host physiology.

5) Metabolic activities of gut bacteria with beneficial effects on host health include the transformation of plant lignans to enterolignans. The production of the latter compounds requires the presence of gut bacteria capable of catalyzing a sequence of four specific reactions. The presence of the respective bacteria and their production of enterolignans exert protective effects in a rat model of breast cancer. This observation offers the opportunity to develop novel concepts of personalized nutrition. Such concepts may include cancer prevention or treatment by the simultaneous application of lignans and the respective bacterial cocktail. However, further studies on the efficacy and safety of such approaches are required to put such concepts into reality.

6) The study on possible prebiotic effects of inulin in pigs did not provide a clear picture about the efficacy of this feed-additive under the selected experimental settings. The same was true for the investigation of anti-inflammatory effects of the probiotic strain *E. faecium* NCIMB 10415 in mice with chronic gut inflammation. It is impossible under these conditions to unravel mechanisms of pre- and probiotic action. Therefore, the identification of robust and reproducible effects of food- and feed-additives on intestinal microbiota and on host health is an important task for future research.

Nine out of the ten studies presented in this thesis have been performed in mice or rats. The use of rodents in nutrition research offers a number of advantages including the possibility to conduct experiments with large numbers of genetically identical animals and the availability of genetically modified models. However, mice and rats do not belong to important “target species” in nutrition research. To benefit from the aforementioned advantages of rodent models in studies on bacteria-diet-host-interactions, mice and rats associated with the complex microbiota from other animal species can be used. In addition, gut bacteria from other species can be used to establish in rodents simplified bacterial communities with selected functions of interest. Those approaches have successfully been applied to the identification of bacterial responses at the molecular level towards different dietary interventions. If host functions are considered important factors in such studies, differences between rodents and higher mammals in e.g. nutrition physiology or gut immunology have to be taken into consideration. In this case it is necessary to verify the relevance of the respective findings in the animal species of interest or to demonstrate the general validity of a given mechanism of host-microbe interactions. This can be brought about by the detection of bacterial genes, proteins or metabolic activities in the intestine of the target animal species.

Taken together, this thesis shows that bacterial communities in the gut are individually composed and that genetically fixed host factors are involved in the selection of an individually composed set of intestinal bacteria. However, it is very likely that the intestinal health status and nutrition are more important for gut microbiota composition than the host genotype. Possible responses of intestinal bacteria towards dietary intervention include the increase of few bacterial species at the expense of formerly dominant gut bacteria. In addition to intestinal microbiota composition, the type of nutrition influences bacterial metabolism according to substrate availability. Moreover, some dietary ingredients modify the intestinal milieu in a way that bacteria have to respond adequately to environmental challenges. There is increasing evidence that those alterations in microbiota composition and function strongly influence host physiology. Since gut bacteria play a role in host health and well-being, strategies have been developed to modulate intestinal microbiota composition and function. However, the application of pre- and probiotics does not always result in clear-cut effects on the bacterial communities in the intestine and beneficial effects on host health are not always observed.

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List of publications and authors' contribution

This thesis is based on the following publications. The authors' contribution is indicated.

PUBLICATION 1

Loh, G., Brodziak, F., Blaut, M. (2008): The Toll-like receptors TLR2 and TLR4 do not affect the intestinal microbiota composition in mice. Environ Microbiol. 10 (3): 709-715.

Concept & study design: Loh

Experimental work: Brodziak, Loh

Data evaluation: Brodziak, Loh

Manuscript preparation: Blaut, Loh

PUBLICATION 2

Wohlgemuth, S., Haller, D., Blaut, M., Loh, G. (2009): Reduced microbial diversity and high numbers of one single *Escherichia coli* strain in the intestine of colitic mice. Environ Microbiol. 11 (6): 1562-1571.

Concept & study design: Loh

Experimental work: Loh, Wohlgemuth

Data evaluation: Loh, Wohlgemuth

Manuscript preparation: Blaut, Haller, Loh, Wohlgemuth

PUBLICATION 3

Wohlgemuth, S., Keller, S., Kertscher, R., Stadion, M., Haller, D., Kisling, S., Jahreis, G., Blaut, M., Loh, G. (2011): Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes 2 (3): 159-166.

Concept & study design: Loh

Experimental work: Jahreis, Kertscher, Kisling, Keller, Loh, Wohlgemuth

Data evaluation: Loh, Wohlgemuth

Manuscript preparation: Blaut, Haller, Loh, Wohlgemuth

PUBLICATION 4

Fleissner, C.K., Huebel, N., Abd El-Bary, M.M., Loh, G., Klaus, S., Blaut, M. (2010): Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr. 104 (6): 919-929.

Concept & study design: Blaut, Klaus, Loh

Experimental work: Abd El-Bary, Fleissner, Huebel, Loh

Data evaluation: Abd El-Bary, Fleissner, Huebel

Manuscript preparation: Blaut, Fleissner, Huebel, Loh

PUBLICATION 5

Becker, N., Kunath, J., Loh, G., Blaut, M. (2011): Human intestinal microbiota: Characterization of a simplified and stable gnotobiotic rat model. Gut Microbes 2 (1): 25-33.

Concept & study design: Blaut, Loh

Experimental work: Becker, Kunath, Loh

Data evaluation: Becker, Kunath

Manuscript preparation: Becker, Blaut, Loh

PUBLICATION 6

Rothe, M., Alpert, C., Engst, W., Musiol, S., Loh, G., Blaut, M. (2012): Impact of nutritional factors on the proteome of intestinal Escherichia coli: Induction of OxyR-dependent proteins AhpF and Dps by a lactose-rich diet. Appl Environ Microbiol 78 (10): 3580-3591.

Concept & study design: Alpert, Blaut, Loh

Experimental work: Rothe, Engst, Loh, Musiol

Data evaluation: Alpert, Rothe, Engst, Loh, Musiol

Manuscript preparation: Alpert, Blaut, Loh, Rothe

PUBLICATION 7

Hrdina, J., Banning, A., Kipp, A., Loh, G., Blaut, M., Brigelius-Flohé, R. (2009): The gastrointestinal microbiota affects the selenium status and selenoprotein expression in mice. J Nutr Biochem. 20 (8): 638-648.

Concept & study design: Banning, Blaut, Brigelius- Flohé, Loh

Experimental work: Banning, Hrdina, Kipp, Loh

Data evaluation: Banning, Brigelius- Flohé, Hrdina, Kipp, Loh

Manuscript preparation: Banning, Blaut, Brigelius- Flohé, Hrdina, Kipp

PUBLICATION 8

Mabrok, H.B., Klopfleisch, R., Ghanem, K., Clavel, T., Blaut, M., Loh, G. (2011): Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model. Carcinogenesis 33 (1): 203-208.

Concept & study design: Blaut, Loh

Experimental work: Loh, Mabrok

Data evaluation: Loh, Mabrok

Manuscript preparation: Blaut, Loh, Mabrok

PUBLICATION 9

Loh, G., Eberhard, M., Brunner, R.M., Hennig, U., Kuhla, S., Kleessen, B., Metges, C.C. (2006): Inulin alters the intestinal microbiota and short chain fatty acid concentrations in growing pigs regardless of their basal diet. J Nutr 136 (5): 1198-1202.

Concept & study design: Hennig, Loh, Metges

Experimental work: Brunner, Eberhard, Hennig, Kleessen, Kuhla, Loh

Data evaluation: Brunner, Eberhard, Hennig, Kleessen, Kuhla, Loh

Manuscript preparation: Loh, Metges

PUBLICATION 10

Ganesh, P.B., Richter, J.F., Blaut, M., Loh, G. (2012): *Enterococcus faecium* NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation. Beneficial Microbes 3 (1): 43-50.

Concept & study design: Blaut, Loh

Experimental work: Ganesh, Loh

Data evaluation: Ganesh, Loh

Manuscript preparation: Blaut, Ganesh, Loh

Complete list of publications (in chronological order):

1. Ganesh, B.P., Klopfleisch, R., **Loh, G.***, Blaut, M. (2013): Commensal *Akkermansia muciniphila* exacerbates gut inflammation in *Salmonella* Typhimurium-infected gnotobiotic mice. Accepted for publication in PLOS ONE (10.1371/journal.pone.0074963). *: Corresponding author.

2. Brodziak, F., Meharg, C., Blaut, M., **Loh, G.** (2013): Differences in mucosal gene expression in the colon of two inbred mouse strains after colonization with commensal gut bacteria. PLOS ONE 8 (8): e72317.

3. Slezak, K., Hanske, L., **Loh, G.**, Blaut, M. (2013): Increased bacterial putrescine has no impact on gut morphology and physiology in gnotobiotic adolescent mice. Benef Microbes 4 (3): 253-266.

4. Wuensch, T., Schulz, S., Ullrich, S., Lill, N., Stelzl, T., Rubio-Aliaga, I., **Loh, G**., Chamaillard, M., Haller, D., Daniel, H. (2013): The peptide transporter PEPT1 is expressed in distal colon in rodents and humans and contributes to water absorption. Am J Physiol Gastrointest Liver Physiol 305 (1): G66-G73.

5. Hanske, L., Engst, W., **Loh, G**., Sczesny, S., Blaut, M., Braune, A. (2013): Contribution of gut bacteria to the metabolism of cyanidin 3-glucoside in human microbiota-associated rats. Br J Nutr 109: 1433-1441.

6. Rothe, M., Alpert, C., **Loh, G.**, Blaut, M. (2013): Novel insights into *E. coli's* hexuronate metabolism: *KduI* facilitates the conversion of galacturonate and glucuronate under osmotic stress conditions. PLOS ONE 8 (2): e56906.

7. **Loh, G.**, Blaut, M. (2012): Role of commensal gut bacteria in inflammatory bowel diseases. Gut Microbes 3 (6): 544-555.

8. Patterson, A.M., Delday, M.I., van Kuppevelt, T.H., **Loh, G**., Blaut, M., Haller, D., Grant, G., Kelly, D. (2012): Expression of heparan sulfate proteoglycans in murine models of experimental colitis. Inflamm Bowel Dis 18 (16): 1112-1126.

9. Rothe, M., Alpert, C., Engst, W., Musiol, S., **Loh, G**., Blaut, M. (2012): Impact of nutritional factors on the proteome of intestinal *Escherichia coli*: Induction of OxyR-dependent proteins AhpF and Dps by a lactose-rich diet. Appl Environ Microbiol 78 (10): 3580-3591.

10. Schumann, S., Alpert, C., Engst, W., **Loh, G**., Blaut, M. (2012): Dextran sodium sulfate-induced inflammation alters the expression of proteins by *Escherichia coli* strains in a gnotobiotic mouse model. Appl Environ Microbiol 78 (5): 1513-1522.

11. Ganesh, B.P., Richter, J.F., Blaut, M., **Loh, G**. (2012): *Enterococcus faecium* NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation. Benef Microbes 3 (1): 43-50.

12. Mabrok, H., Klopfleisch, R., Ghanem, K., Clavel, T., Blaut, M., **Loh, G**. (2012): Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model. Carcinogenesis 33 (1): 203-208.

13. Matthies, A., **Loh, G.**, Blaut, M., Braune, A. (2012): Daidzein and genistein are converted to equol and 5-hydroxy-equol by human intestinal *Slackia isoflavoniconvertens* in gnotobiotic rats. J Nutr 142 (1): 40-46.

14. Wohlgemuth, S., Keller, S., Kertscher, R., Stadion, M., Haller, D., Kisling, S., Jahreis, G., Blaut, M., **Loh, G**. (2011): Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes 2 (3): 159-166.

15. Becker, N., Kunath, J., **Loh, G**., Blaut, M. (2011): Human intestinal microbiota: Characterization of a simplified and stable gnotobiotic rat model. Gut Microbes 2 (1): 25-33.

16. Gibson, G.R., Scott, K.P., Rastall, R.A., Tuohy, K.M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E.F., Saulnier, D., **Loh, G**., Macfarlane, S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkoop, I., Walker, C., Buddington, R. (2010): Dietary prebiotics: current status and new definition. Food Sci Tech Bull Funct Foods 7 (1): 1-19.

17. Hanske, L., **Loh, G**., Sczensny, S., Blaut, M., Braune, A. (2010): Recovery and metabolism of xanthohumol in germ-free and human microbiota-associated rats. Mol Nutr Food Res 54: 1405-1413.

18. Hörmannsperger, G., Clavel, T., Hoffmann, M., Reiff, C., Kelly, D., **Loh, G**., Blaut, M., Hölzwimmer, G., Haller, D. (2010): Posttranslational inhibition of proinflammatory chemokine secretion in intestinal epithelial cells: Implications for specific IBD indications. J Clin Gastroenterol 44 (Suppl. 1): S10-S15.

19. Vogel-Scheel, J., Alpert, C., Engst, W., **Loh, G**., Blaut, M. (2010): Requirement of purine and pyrimidine synthesis for colonization of the mouse intestine by *Escherichia coli*. Appl Environ Microbiol 76 (15): 5181-5187.

20. Fleissner, C.K., Huebel, N., Abd El-Bary, M.M., **Loh, G**., Klaus, S., Blaut, M. (2010): Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr 104 (6): 919-929.

21. Woting, A., Clavel, T., **Loh, G**., Blaut, M. (2010): Bacterial transformation of dietary lignans in gnotobiotic rats. FEMS Microbiol Ecol 72 (3): 507-514.

22. Wohlgemuth, S., **Loh, G**., Blaut, M. (2010): Recent developments and perspectives in the investigation of probiotic effects. Int J Med Microbiol 300 (1): 3-10. (invited review)

23. Reiff, C., Delday, M., Rucklidge, G., Reid, M., Duncan, G., Wohlgemuth, S., Hörmannsperger, G., **Loh, G**., Blaut, M., Collie-Duguid, E., Haller, D., Kelly, D. (2009): Balancing inflammatory, lipid, and xenobiotic signaling pathways by VSL#3, a biotherapeutic agent, in the treatment of inflammatory bowel disease. Inflamm Bowel Dis 15 (11): 1721-1736.

24. Hanske, L., **Loh, G**., Sczesny, S., Blaut, M., Braune, A. (2009): The bioavailability of apigenin-7-glucoside is influenced by human intestinal microbiota in rats. J Nutr 139 (6): 1095-1102.

25. Martin, F.P., Rezzi, S., Philippe, D., Tornier, L., Messlik, A., Hölzlwimmer, G., Baur, P., Quintanilla-Fend, L., **Loh, G**., Blaut, M., Blum, S., Kochhar, S., Haller, D. (2009): Metabolic assessment of gradual development of moderate experimental colitis in IL-10 deficient mice. J Proteome Res 8 (5): 2376-2387.

26. Wohlgemuth, S., Haller, D., Blaut, M., **Loh, G**. (2009): Reduced microbial diversity and high numbers of one single *Escherichia coli* strain in the intestine of colitic mice. Environ Microbiol 11 (6): 1562-1571.

27. Hoermannsperger, G., Clavel, T., Hoffmann, M., Reiff, C., Kelly, D., **Loh, G**., Blaut, M., Hölzlwimmer, G., Laschinger, M., Haller, D. (2009): Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. PLOS ONE 4 (2): e4365.

28. Alpert, C., Scheel, J., Engst, W., **Loh, G**., Blaut, M. (2009): Adaptation of protein expression by Escherichia coli in the gastrointestinal tract of gnotobiotic mice. Environ Microbiol 11 (4): 751-761.

29. Hrdina, J., Banning, A., Kipp, A., **Loh, G**., Blaut, M., Brigelius-Flohé, R. (2009): The gastrointestinal microbiota affects the selenium status and selenoprotein expression in mice. J Nutr Biochem 20 (8): 638-648.

30. **Loh, G**., Brodziak, F., Blaut, M. (2008): The Toll-like receptors TLR2 and TLR4 do not affect the intestinal microbiota composition in mice. Environ Microbiol 10 (3): 709-715.

31. **Loh, G**., Eberhard, M., Brunner, R.M., Hennig, U., Kuhla, S., Kleessen, B., Metges, C.C. (2006): Inulin alters the intestinal microbiota and short chain fatty acid concentrations in growing pigs regardless of their basal diet. J Nutr 136 (5): 1198-1202.

Annex

PUBLICATION 1

Loh, G., Brodziak, F., Blaut, M. (2008): The Toll-like receptors TLR2 and TLR4 do not affect the intestinal microbiota composition in mice. Environ Microbiol. 10 (3): 709-715.

DOI: 10.1111/j.1462-2920.2007.01493.x

Abstract: The interaction between intestinal epithelial cells and microbes is partly mediated by Toll-like receptors (TLRs). Sensing of Gram-positive and Gram-negative bacteria by TLR2 and TLR4, respectively, can result in immune system activation and in an exclusion of bacteria from the intestine. To test the impact of these TLRs on bacterial composition, germ-free TLR2/TLR4 double knock-out mice and the corresponding C57BL/10ScSn wild-type mice where associated with fecal bacteria from one single donor mouse. In addition, C3H/HeOuJ and BALB/c mice were used in this study. Fecal bacteria were monitored over 13 weeks with denaturing-gradient gel electrophoresis (DGGE). Colonic bacteria were enumerated by fluorescent in situ hybridization (FISH) and short-chain fatty acids (SCFA) were measured in cecal samples. No effect of the TLRs on intestinal microbiota composition and SCFA concentrations was observed. However, the microbiota composition as reflected by DGGE band patterns differed between C3H and BALB/c mice on the one hand and C57BL/10 mice on the other hand. Corresponding differences between the mouse strains were also observed in cecal propionic, valeric and i-valeric acid concentrations. No differences between the animals were observed in the numbers of bacteria detected by FISH. We conclude that genetic traits but not TLR2 and TLR4 have an impact on the intestinal microbiota composition.

PUBLICATION 2

Wohlgemuth, S., Haller, D., Blaut, M., Loh, G. (2009): Reduced microbial diversity and high numbers of one single Escherichia coli strain in the intestine of colitic mice. Environ Microbiol. 11 (6): 1562-1571.

DOI: 10.1111/j.1462-2920.2009.01883.x.

Abstract: Commensal bacteria play a role in the aetiology of inflammatory bowel diseases (IBD). High intestinal numbers of *Escherichia coli* in IBD patients suggest a role of this organism in the initiation or progression of chronic gut inflammation. In addition, some *E. coli* genotypes are more frequently detected in IBD patients than others. We aimed to find out whether gut inflammation in an IBD mouse model is associated with a particular *E. coli* strain. Intestinal contents and tissue material were taken from 1-, 8-, 16- and 24-week-old interleukin 10-deficient (IL-$10^{-/-}$) mice and the respective wild-type animals. Cecal and colonic inflammation was observed in IL-$10^{-/-}$ animals from the 8 weeks of life on accompanied by a lower intestinal microbial diversity than in the respective wild-type animals. Culture-based and molecular approaches revealed that animals with gut inflammation harboured significantly higher numbers of *E. coli* than healthy controls. Phylogenetic grouping according to the *E. coli* Reference Collection (ECOR) system and strain typing by random-amplified polymorphic DNA and pulsed-field gel electrophoresis revealed that all mice were colonized by one single *E. coli* strain. The strain was shown to have the O7:H7:K1 serotype and to belong to the virulence-associated phylogenetic group B2. In a co-association experiment with gnotobiotic mice, the strain outnumbered *E. coli* ECOR strains belonging to the phylogenetic group A and B2 respectively. A high number of virulence- and fitness-associated genes were detected in the strain's genome possibly involved in the bacterial adaptation to the murine intestine.

PUBLICATION 3

Wohlgemuth, S., Keller, S., Kertscher, R., Stadion, M., Haller, D., Kisling, S., Jahreis, G., Blaut, M., Loh, G. (2011): Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes 2 (3): 159-166.

DOI: 10.4161/GMIC.2.3.16104.

Abstract: Reduced gut microbiota diversity in conjunction with a bloom of few bacterial species is a common feature in inflammatory bowel disease (IBD) patients. However, the environmental changes caused by inflammation and their possible impact on the microbiota are largely unknown. Since IBD is associated with an impaired intestinal steroid metabolism, we hypothesized that changes in intestinal steroid and particularly bile acid (BA) concentrations affect microbial communities. We used Interleukin-10 deficient (IL-$10^{-/-}$) mice as a model for chronic gut inflammation. Healthy wild-type mice served as controls. In these animals, intestinal steroid concentrations and gut microbial diversity were analyzed at 24 weeks of age. The IL $10^{-/-}$ mice developed moderate inflammation in cecum and colon and colorectal tumor formation was observed in 55 % of the animals. Compared to the healthy conditions, gut inflammation was associated with higher intestinal cholesterol and cholic acid concentrations and a reduced microbial diversity. The latter was accompanied by a proliferation of *Robinsoniella peoriensis*, *Clostridium innocuum*, *Escherichia coli*, and *Enterococcus gallinarum*. All these species proved to be highly bile acid resistant. We concluded that chronic colitis in IL-$10^{-/-}$ mice is associated with changes in intestinal steroid profiles. These changes may be due to alterations in gut microbiota composition or vice versa. Whether the bacterial sterol and bile acid metabolism is implicated in colitis and colorectal carcinoma etiology remains to be clarified.

PUBLICATION 4

Fleissner, C.K., Huebel, N., Abd El-Bary, M.M., Loh, G., Klaus, S., Blaut, M. (2010): Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr. 104 (6): 919-929.

DOI: 10.1017/S0007114510001303.

Abstract: The gut microbiota has been implicated in host nutrient absorption and energy homeostasis. We studied the influence of different diets on body composition in germ-free (GF) and conventional (CV) mice. GF and CV male adult C3H mice were fed ad libitum a semi-synthetic low-fat diet (LFD; carbohydrate-protein-fat ratio: 41:42:17; 19.8 kJ/g), a high-fat diet (HFD; 41:16:43; 21.4 kJ/g) or a commercial Western diet (WD; 41:19:41; 21.5 kJ/g). There was no difference in body weight gain between GF and CV mice on the LFD. On the HFD, GF mice gained more body weight and body fat than CV mice, and had lower energy expenditure. GF mice on the WD gained significantly less body fat than GF mice on the HFD. GF mice on both HFD and WD showed increased intestinal mRNA expression of fasting-induced adipose factor/angiopoietin-like protein 4 (Fiaf/Angptl4), but they showed no major changes in circulating Fiaf/Angptl4 compared with CV mice. The fecal microbiota composition of the CV mice differed between diets: the proportion of Firmicutes increased on both HFD and WD at the expense of the Bacteroidetes. This increase in the Firmicutes was mainly due to the proliferation of one family within this phylum: the Erysipelotrichaceae. We conclude that the absence of gut microbiota does not provide a general protection from diet-induced obesity, that intestinal production of Fiaf/Angptl4 does not play a causal role in gut microbiota-mediated effects on fat storage and that diet composition affects gut microbial composition to larger extent than previously thought.

PUBLICATION 5

Becker, N., Kunath, J., Loh, G., Blaut, M. (2011): Human intestinal microbiota: Characterization of a simplified and stable gnotobiotic rat model. Gut Microbes 2 (1): 25-33.

DOI: 10.4161/gmic.2.1.14651.

Abstract: The study of host microbe interactions is hampered by the complexity and inter-individual variability of the human gut microbiota. Therefore, a simplified human intestinal microbiota (SIHUMI) consisting of seven bacterial species was introduced into germfree rats. Species selection was based on numerical importance and fermentative abilities in the human gut. Association of the rats with the SIHUMI (*Anaerostipes caccae*, *Bacteroides thetaiotaomicron*, *Bifidobacterium longum*, *Blautia producta*, *Clostridium ramosum*, *Escherichia coli* and *Lactobacillus plantarum*) resulted in increased fecal concentrations of short chain fatty acids compared to germfree animals. Since the fecal butyrate concentration was low (0.9 ± 0.5 µmol/g dry matter) the SIHUMI was complemented with *Clostridium butyricum*. This extended bacterial community (SIHUMIx) led to an increased fecal butyrate concentration of 1.5 ± 0.7 µmol/g dry matter. Besides forming SCFA, the SIHUMIx was capable of degrading mucins, β-aspartylglycine and bilirubin. These features are characteristic of conventional animals but not observed in germfree animals. Dietary interventions with modifications in fibre and fat content led to changes in the proportion of community members. The relative increase of one member of this community in response to a high-fat diet reflects the situation reported for obese mice and human subjects. The strength of the model communities is their remarkable stability over time and their easy transfer to the offspring.

PUBLICATION 6

Rothe, M., Alpert, C., Engst, W., Musiol, S., Loh, G., Blaut, M. (2012): Impact of nutritional factors on the proteome of intestinal *Escherichia coli*: Induction of OxyR-dependent proteins AhpF and Dps by a lactose-rich diet. Appl Environ Microbiol 78 (10): 3580-3591.

DOI: 10.1128/AEM.00244-12.

Abstract: To study the impact of nutritional factors on protein expression of intestinal bacteria, gnotobiotic mice monoassociated with *Escherichia coli* K-12 were fed three different diets: a diet rich in starch, a diet rich in nondigestible lactose, and a diet rich in casein. Two-dimensional gel electrophoresis and electrospray-tandem mass spectrometry were used to identify differentially expressed proteins of bacteria recovered from small intestine and cecum. Oxidative stress response proteins such as AhpF, Dps, and Fur, all of which belong to the oxyR regulon, were upregulated in *E. coli* isolates from mice fed the lactose-rich diet. Luciferase reporter gene assays demonstrated that osmotic stress caused by carbohydrates led to the expression of ahpCF and dps, which was not observed in an E. coli ΔoxyR mutant. Growth of ahpCF and oxyR deletion mutants was strongly impaired when nondigestible sucrose was present in the medium. The wild-type phenotype could be restored by complementation of the deletions with plasmids containing the corresponding genes and promoters. The results indicate that some OxyR-dependent proteins play a major role in the adaptation of *E. coli* to osmotic stress. We conclude that there is an overlap of osmotic and oxidative stress responses. Mice fed the lactose-rich diet possibly had a higher intestinal osmolality, leading to the upregulation of OxyR-dependent proteins, which enable intestinal E. coli to better cope with diet-induced osmotic stress.

PUBLICATION 7

Hrdina, J., Banning, A., Kipp, A., Loh, G., Blaut, M., Brigelius-Flohé, R. (2009): The gastrointestinal microbiota affects the selenium status and selenoprotein expression in mice. J Nutr Biochem. 20 (8): 638-648.

DOI: 10.1016/j.jnutbio.2008.06.009

Abstract: Colonization of germ-free (GF) mice has been shown to induce the gastrointestinal form of the selenium-dependent glutathione peroxidases, GPx2. Since bacterial colonization of the gastrointestinal tract is associated with stress, we aimed to clarify how bacteria affect selenoprotein expression in unstressed conditions. GF and conventional (CV) FVB/NHan(TMHsd) mice were fed a selenium-poor (0.086 ppm) or a selenium-adequate (0.15 ppm) diet for 5 weeks starting from weaning. Each group consisted of five animals. Specific glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) expression was measured in plasma, liver and intestinal sections by activity, protein and mRNA level as appropriate. Under selenium-adequate conditions, selenoprotein expression did not differ in GF and CV mice. Under selenium-limiting conditions, however, GF mice generally contained higher GPx and TrxR activities in the intestine and liver, higher GPx1 protein and RNA levels in the liver, higher GPx2 protein levels in the proximal and distal jejunum and colon and higher GPx1 and GPx2 RNA levels in the colon. In addition, higher selenium concentrations were estimated in plasma, liver and cecum. All differences were significant. It is concluded that bacteria may compete with the host for selenium when availability becomes limiting. A variable association with different microorganisms might influence the daily requirement of mice for selenium. Whether the microbiota also affects the human selenoprotein status appears worthy of investigation.

PUBLICATION 8

Mabrok, H., Klopfleisch, R., Ghanem, K., Clavel, T., Blaut, M., Loh, G. (2011): Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model. Carcinogenesis 33 (1): 203-208.

DOI: 10.1093/carcin/bgr256.

Abstract: High dietary lignan exposure is implicated in a reduced breast cancer risk in women. The bacterial transformation of plant lignans to enterolignans is thought to be essential for this effect. To provide evidence for this assumption, gnotobiotic rats were colonized with the lignan-converting bacteria *Clostridium saccharogumia*, *Eggerthella lenta*, *Blautia producta* and *Lactonifactor longoviformis* (LCC rats). Germ-free rats were used as the control. All animals were fed a lignan-rich flaxseed diet and breast cancer was induced with 7,12-dimethylbenz(a)anthracene. The lignan secoisolariciresinol diglucoside was converted into the enterolignans enterodiol and enterolactone in the LCC but not in the germ-free rats. This transformation did not influence cancer incidence at the end of the 13 weeks experimental period but significantly decreased tumor numbers per tumor-bearing rat, tumor size, tumor cell proliferation and increased tumor cell apoptosis in LCC rats. No differences between LCC and control rats were observed in the expression of the genes encoding the estrogen receptors (ERs) α, ERβ and G-coupled protein 30. The same was true for IGF-1 and EGFR involved in tumor growth. The activity of selected enzymes involved in the degradation of oxidants in plasma and liver was significantly increased in the LCC rats. However, plasma and liver concentrations of reduced glutathione and malondialdehyde, considered as oxidative stress markers, did not differ between the groups. In conclusion, our results show that the bacterial conversion of plant lignans to enterolignans beneficially influences their anticancer effects.

PUBLICATION 9

Loh, G., Eberhard, M., Brunner, R.M., Hennig, U., Kuhla, S., Kleessen, B., Metges, C.C. (2006): Inulin alters the intestinal microbiota and short chain fatty acid concentrations in growing pigs regardless of their basal diet. J Nutr 136 (5): 1198-1202.

Available at: http://jn.nutrition.org/content/136/5/1198.full.pdf

Abstract: Inulin stimulates intestinal bifidobacteria in humans and rodents but its effect in pigs is inconsistent. We assessed the effect of inulin on the intestinal microbiota by fluorescent in situ hybridization in growing pigs (age 9-12 wk). Pigs (n = 64) were assigned to 2 types of basal diets [wheat and barley (WB) or corn and wheat gluten (CG)] with or without 3% inulin (WBI or CGI) for 3 and 6 wk (n = 8/group) to test whether naturally occurring dietary fibers influence the inulin effect. Intestinal organic acids, pH values, and residual inulin were determined. The composition of the microbiota was highly individual. The duration of feeding did not affect any of the variables tested; therefore, data for the 2 periods were pooled. Bifidobacteria were detected in less than half of the pigs. Inulin did not stimulate lactobacilli and bifidobacteria numbers irrespective of the basal diet, although 20-50% of inulin was degraded in the jejunum. The number of pigs with colonic bifidobacteria was higher in those fed diets containing inulin (40 vs. 13%; P < 0.05). Total colonic short-chain fatty acid (SCFA) concentrations were lower in both inulin-fed groups due to reduced acetate (P < 0.05). Proportions of colonic butyrate were higher in pigs fed inulin-supplemented diets (P < 0.05). Colonic pH tended to be lower in the WB groups (WB; 6.6 +/- 0.6), and was higher due to inulin (CGI, 7.1 +/- 0.1; P < 0.05). In conclusion, inulin affected intestinal SCFA and the number of pigs harboring bifidobacteria; this effect was independent of the basal diet.

PUBLICATION 10

Ganesh, P.B., Richter, J.F., Blaut, M., Loh, G. (2012): *Enterococcus faecium* NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation. Beneficial Microbes 3 (1): 43-50.

DOI: 10.3920/BM2011.0050.

Abstract: *Enterococcus faecium* NCIMB 10415 reduces diarrhoea incidence and duration in animals and human study subjects. We tested whether the strain is also capable of reducing chronic gut inflammation and aimed to identify mechanisms that are involved in possible probiotic effects. To identify health-promoting mechanisms of the strain, we used interleukin-10-deficient mice that spontaneously develop gut inflammation and fed these mice a diet containing NCIMB 10415 for 3, 8 and 24 weeks, respectively. Control mice were fed a diet which was identically composed but did not contain the strain. After 3 weeks of intervention the experimental animals were less inflamed in the cecum than the control animals. This effect was not observed in the colon and there were no differences between experimental and control mice at any other time point. The application of the strain was associated with higher expression levels of interferon gamma and interferon gamma-induced protein 10 after 3 and 24 but not after 8 weeks of feeding. No differences between the animals were observed in intestinal barrier function or intestinal microbiota composition. However, we observed a low abundance of the mucin-degrading bacterium *Akkermansia muciniphila* in the mice that were fed NCIMB 10415 for 8 weeks. These low cell numbers were associated with a significantly lower cecal inflammation score and improved paracellular permeability as compared to the NCIMB-treated mice that were killed after 3 and 24 weeks of intervention. In conclusion, NCIMB 10415 is not capable of reducing gut inflammation in the IL-$10^{-/-}$ mouse model. The exact role of *A. muciniphila* and of a possible interaction between this bacterium, NCIMB 10415 and the host in gut inflammation requires further investigation.