1 GENERAL INTRODUCTION AND WORK HYPOTHESIS

1.1 GENERAL INTRODUCTION

The human intestinal microbial ecosystem plays an important role in maintaining health. A multitude of diseases including e.g. diarrhea, cancer, gastrointestinal inflammatory disorders such as necrotizing enterocolitis (NEC) of neonates, or obesity has been shown to be linked to microbial composition and metabolic activity. As dietary composition determines the substrates available for the intestinal microbiota, thereby affecting their composition and activity, dietary modulation appears to be an efficient and promising tool to improve host health by beneficially steering microbial composition and metabolism (Govers et al., 1999, Duncan et al., 2008). Within this regard, health benefits of dietary fiber have frequently been described and acknowledged. Higher intakes of dietary fiber are linked to lower risk of cardiovascular disease, protective effect against the development of colon cancer and lower body weight gain (Slavin, 1987). On the other hand, especially so-called modern ways of nutrition such as fast food consumption, usually low in dietary fiber but high in fat and sugar, contribute to the development of metabolic diseases such as obesity. In 2014, more than 1.9 billion adults, 18 years and older, were overweight (WHO, 2015). Of these, over 600 million were obese. Therefore, research on potential interactions of diet and gut microbial composition and activity is being accomplished. Concerning animal models for the human, rodents still belong to most frequently used model organisms. However, the physiological similarity between humans and pigs in terms of digestive and associated metabolic processes puts the pig in a superior position over other non-primate models. The pig is increasingly recognized as suitable animal model for the human, as pigs are human sized omnivorous animals with nutritional requirements similar to those of humans. In several studies, the pig has been used as model to assess microbiota-health-interactions, since pigs exhibit similar syndromes as humans, such as NEC and weanling diarrhea (Siggers et al., 2008, Shu et al., 2001). Besides, in other cases including obesity, studies with mice and humans have shown that the composition and metabolic activity of the gut microbiota is closely associated with the development of the disease (Ley et al., 2005, Turnbaugh et al., 2009, Schwiertz et al. 2010), though there exist inconsistencies in results between studies.

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1.2 WORK HYPOTHESIS

Based on a comprehensive literature review (Chapter 2), it appears that there is a considerable need for animal models to study potential ways of influencing health by dietary means based on the present knowledge about the interactions between gut microbiota, health and disease development. Still, rodents dominate as model animals, mainly due to the considerable lower costs for keeping and handling in comparison to pigs. There is a scarcity of information, however, on the suitability of the pig for assessing the development of the gut microbiota and microbial activity upon feeding of diets, which have a similar composition compared to those frequently used in human nutrition. Thus, two different types of diets, varying substantially in fat and fiber content, were fed in a long-term feeding trial to growing pigs. The high-fat diet, also referred to as Western style diet, was high in fat and sugar content, whereas the high-fiber diet, sometimes in the literature also referred to as Mediterranean diet, was mainly composed of ingredients high in fiber content. It was hypothesized that these differences in diet composition will be reflected in microbiota composition and its metabolic activity. Molecular-based methods (real-time qPCR) in addition to advanced metaproteomic- and fingerprint analyses were used to determine diet-induced changes in fecal microbiota composition (Chapter 3). These analyses were complemented by measurements of microbiota composition and microbial metabolites in cecal and colonic digesta, in addition to determination of pigs' carcass traits and biochemical markers in the blood sera (Chapter 4).

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CHAPTER 2

USE OF PIGS AS A POTENTIAL MODEL FOR RESEARCH INTO DIETARY MODULATION OF THE HUMAN GUT MICROBIOTA

Sonja N. Heinritz, Rainer Mosenthin and Eva Weiss

Institute of Animal Nutrition, University of Hohenheim, 70593 Stuttgart, Germany

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2 USE OF PIGS AS A POTENTIAL MODEL FOR RESEARCH INTO DIETARY MODULATION OF THE HUMAN GUT MICROBIOTA

2.1 SUMMARY

The human intestinal microbial ecosystem plays an important role in maintaining health. A multitude of diseases including diarrhoea, gastrointestinal inflammatory disorders, such as necrotising enterocolitis (NEC) of neonates, and obesity are linked to microbial composition and metabolic activity. Therefore, research on possible dietary strategies influencing microbial composition and activity, both preventive and curative, is being accomplished. Interest has focused on pre- and probiotics that stimulate the intestinal production of beneficial bacterial metabolites such as butyrate, and beneficially affect microbial composition. The suitability of an animal model to study dietary linked diseases is of much concern. The physiological similarity between humans and pigs in terms of digestive and associated metabolic processes places the pig in a superior position over other non-primate models. Furthermore, the pig is a human-sized omnivorous animal with comparable nutritional requirements, and shows similarities to the human intestinal microbial ecosystem. Also, the pig has been used as a model to assess microbiota-health interactions, since pigs exhibit similar syndromes to humans, such as NEC and partly weanling diarrhoea. In contrast, when using rodent models to study diet-microbiota-health interactions, differences between rodents and humans have to be considered. For example, studies with mice and human subjects assessing possible relationships between the compositionand metabolic activity of the gut microbiota and the development of obesity have shown inconsistencies in results between studies. The present review displays the similarities and differences in intestinal microbial ecology between humans and pigs, scrutinising the pig as a potential animal model, with regard to possible health effects.

2.2 INTRODUCTION

The importance of the intestinal microbiota for gastrointestinal (GI) functions and health has been shown in many studies with human subjects, but also with model animals such as mice and pigs (1,2). In addition, several disease patterns in humans may be associated with the composition and/or metabolic activity of the intestinal microbiota. Relationships between variations in the abundance or metabolic activity of certain phyla and bacterial

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groups and the development of several medical conditions, such as obesity, have been established (1,3). Other diseases related to changes in the composition and activity of the intestinal microbiota include diarrhoea and necrotising enterocolitis (NEC) (4,5). As diet composition reflects the substrates available for the intestinal microbiota, thereby affecting their composition and metabolic activity, dietary modulation appears to be a valuable and promising tool to improve host health by beneficially steering microbial composition and metabolism (6,7). Therefore, dietary supplementation of food additives, such as probiotics, has been frequently proposed. Probiotic food supplements, i.e. viable micro-organisms, may alter the microbiota of the host, thus beneficially influencing its health (8), with *Lactobacillus* and *Bifidobacterium* species being among frequently used probiotics (9). Additionally, non-digestible food ingredients such as oligosaccharides can be used as prebiotics to modulate the gut microbiota, as they have proven to stimulate the growth and/or activity of beneficial bacterial groups in the colon (10).

In the past, rodents have been used most frequently as animal models. However, despite some advantages, such as their low costs in breeding, feeding and handling, several physiological and metabolic differences compared with humans have to be acknowledged. These differences include rodents being originally granivore animals in contrast to omnivorous humans, with fermentation taking place in their large caecum, while also practicing caecotrophy (11,12). Furthermore, the rat as a small animal needs more feed per unit body weight (BW), which means a faster digesta passage rate and, in addition, often a lower capacity for fibre digestion compared with humans (13). With respect to gut microbiota, the main bacterial groups such as Firmicutes and Bacteroidetes present in the faecal and caecal contents of rats and mice are similar compared with humans (14-16). However, the abundance of important bacteria genera such as *Lactobacillus* and *Bifidobacterium* spp. differs between humans and rats (17-19).

The pig as a human-sized, omnivorous animal with anatomical and physiological similarities to humans has been proposed as an alternative animal model for research into dietary modulation of the human gut microbiota (20,21). Similar to humans, the gut microbiota of pigs mainly consists of the Firmicutes and Bacteroidetes phyla (22). In this context, it has to be mentioned that the pig has already been used for a long time as an animal model for research into human nutrition and biomedicine (23), as it has been described in several reviews comparing pigs with other animal models (24). According to these authors, there are diverse areas of application for the pig model including amino acid metabolism, total

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parenteral nutrition, rotavirus infection, and bacterial and viral pneumonia. Puiman & Stoll (25) reviewed the use of animal models to study neonatal nutrition in humans. They concluded that neonatal mice are suitable for mechanistic and genomic research in postnatal nutrition and associated diseases, while the neonatal pig is a suitable model to investigate acute and chronic effects of parenteral and enteral nutrition on whole-body metabolism in addition to specific tissues.

In order to assess the suitability of the pig as an appropriate model animal, a systematic comparison of the gut microbiota of pigs and humans is inevitable. In the present review, anatomical and physiological similarities and differences between the GI tract (GIT) of pigs and humans will be described, with special focus on the composition and metabolic activities of the microbiota harboured in the GIT of these two species. Studies in which the pig was used as a model to assess the role of the intestinal microbiota in disease development will be reviewed and, in particular, the impact of diet composition on the intestinal microbial community and its metabolic activity will be evaluated to identify beneficial effects on host health and recovery.

2.3 ANATOMY OF THE GASTROINTESTINAL TRACT AND BODY CONSTITUTION – SIMILARITIES AND DIFFERENCES BETWEEN HUMANS AND PIGS

The porcine and human intestinal tracts are very similar with respect to anatomical and physiological characteristics (23,26,27). This includes comparable digesta transit times (23) and analogous digestive and absorptive processes (12). Moreover, the minimum nutrient requirements of pigs are similar to recommended daily allowances of humans when expressed per kg of dietary DM (23). Additionally, when calculating the relationship between intestinal length and BW, intestinal length amounts to 0.1 m/kg BW, for both humans and pigs (28). However, the two species differ in the total length of their intestinal tracts. In humans, the length of the small and large intestines is 5.5-7 m and 1.5 m, respectively, at maturity (33 years) (29), whereas the corresponding values in pigs amount to 15-22 and 4-6 m, respectively, at an assumed maturity age of 3 years (23,30). Other anatomical differences include a more distinct separation between duodenum, jejunum and ileum in humans compared with pigs, and a different arrangement of the small and large intestines in the abdomen of the two species (31).

When using pigs as an animal model for humans, the rapid growth and mature size of modern swine breeds (90-120 kg and 330-450 kg at the age of 6 months and at the adult stage,

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respectively) to obtain maximal performance have to be taken into account when comparing the two species (32). With regard to their body size, mini-pigs, with an adult BW of only 70-120 kg, might be closer to humans, while also being easier to handle, though more expensive (33).

2.4 INTESTINAL MICROBIOTA OF HUMANS AND PIGS: MICROBIAL FERMENTATION AND COMPOSITION

Both pigs and humans are colon fermenters, and they have a similar composition of the colonic microbiota (23). However, symbiotic micro-organisms harboured in the GIT play a relatively minor role in the *de novo* synthesis of nutrients such as amino acids and fatty acids compared with ruminants (33). Pigs exhibit significant caecal fermentation (34), and may obtain up to 30% of their energy requirement for maintenance from microbially produced SCFA in the large intestine (35). On the contrary, humans lack a distinct caecum (36), and only about 7% of their energy requirement for maintenance originates from SCFA produced in the colon (37). In the GIT, SCFA and various gases (H_2, CO_2) are the major metabolites produced by microbial fermentation (38). The largest fraction of SCFA is acetate, propionate and butyrate, with acetate being the most prominent of the three major SCFA, making up approximately two-thirds of the total SCFA (39). Acetate is extensively produced by various bacterial groups, while propionate and butyrate, which are known for their beneficial effects on the host (40), are produced by a limited number of bacterial groups only. For example, propionate is largely metabolised in the liver where it is used as a precursor for gluconeogenesis and may inhibit lipogenesis (41,42), while butyrate is the preferred energy source for the colonic epithelial cells (43). Common butyrate producers in the GIT are clostridia such as *Roseburia* spp. and *Eubacterium rectale* (44). Increased concentrations of SCFA in the GIT, especially acetate, are suggested to secure a preventive effect on the overgrowth of endogenous *Escherichia coli* (45).

2.4.1 THE HUMAN GUT MICROBIOTA: A BRIEF OVERVIEW

The composition of the intestinal microbiota in humans is extremely complex and therefore difficult to describe, and there exist plenty of data. The following section aims to provide a rather general and brief overview, thereby focusing on the major genera.

The human GIT contains about 10^{14} bacterial cells, with the highest density and diversity present in the large intestine (46,47). These bacteria belong to the Firmicutes group

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(about 60 %), Bacteroidetes (about 15 %), followed by Actinobacteria (about 15 %), Verrucomicrobia (about 2 %), Proteobacteria (about 1%) and Methanobacteriales (about 1%) (48,49). In humans, the phylum Firmicutes comprises species belonging to the genera *Eubacterium*, *Clostridium*, *Ruminococcus* and *Butyrivibrio* (50) with the *Eubacterium rectale-Clostridium coccoides* group being represented in large numbers of total bacteria, accounting for about 28% of total bacteria in faecal samples (Table 1) (17). Bacteroidetes are represented by genera related to *Bacteroides*, which are generally present in high numbers in the human gut microbiota, averaging 9 to 42% of total bacteria (51). Actinobacteria, the third most prevailing phylum in the human GIT, comprise the *Collinsella-Atopobium* group, with 0.3-3.7% of total bacteria (52,53), and bifidobacteria. Bifidobacteria are known for their health-promoting properties (10), and are used as probiotic food ingredients (54), such as *Bifidobacterium bifidum* and *Bifidobacterium longum* (55). They compose about 4% of the human faecal microbiota (17,53).

Table 1: Proportions of bacteria in human faeces assessed by fluorescent *in situ* hybridisation combined with flow cytometry detection* (Mean values and standard deviations)

Probe	Proportions of total bacteria in faecal sample (%)	SD
Clostridium coccoides-Eubacterium rectale (Erec 482)	28.0†	11.3
	22.0‡	7.6
Clostridium leptum (Clep 866)	25.2†	7.6
	21.7§	7.7
Bacteroides (Bac 303)	8.5†	7.1
	9.1‡	6.7
Atopobium (Ato291)	3.1†	2.8
	3.7‡	2.8
Bifidobacterium (Bif 164)	4.4†	4.3
	4.1‡	3.9
Lactobacillus-Enterococcus (Lab 158)	1.8†	1.4
	2.0§	1.3
Enterobacteria (Enter 1432)	1.0‡	2.8
	0.1§	0.1
Streptococcus (Strc 493)	0.6†	0.8
	0.4§	0.6

*Donors on Western European Diet.
†Data compiled according to Lay *et al.* (2005a) (17). Age of donors: 7-52 years (*n*91).
‡Data compiled according to Rigottier-Gois *et al.* (2003) (53). Age of donors: 3-68 years (*n*23).
§Data compiled according to Lay *et al.* (2005b) (56). Age of donors: 25-45years (*n*21).

According to the results of representative studies as outlined in Table 1, lactobacilli may be present in colonic or faecal contents of humans to a lower extent, with levels comprising

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about 2.0% of total bacteria (17,56), or less (0.2-1.0 %) (57). However, there exists a considerable variation between individuals as well (58). Streptococci in the human microbiota occur in a similar range as lactobacilli, amounting to 0·4-1·6% of total bacteria (17,56,59).

2.4.2 THE PORCINE GUT MICROBIOTA

Similar to humans, the gut microbiota of pigs mainly consists of the Firmicutes and Bacteroidetes phyla (22). The main bacterial groups in the pig GIT comprise the following bacteria: *Streptococcus* spp., *Lactobacillus* spp., *Eubacterium* spp., *Fusobacterium* spp., *Bacteroides* spp., *Peptostreptococcus* spp., *Bifidobacterium* spp., *Selenomonas* spp., *Clostridium* spp., *Butyrivibrio* spp., *Escherichia* spp., *Prevotella* and *Ruminococcus* spp. (22,60 - 65). Kim *et al.* (66) assessed the pig's microbiota in faecal samples, which were collected five times in 3-week intervals starting at the age of 10 weeks. According to their results, the two most abundant bacterial genera of the pigs are *Prevotella* spp. (11.6% of total bacteria), belonging to the Bacteroidetes phylum, and *Anaerobacter* (10.4 %), members of the Firmicutes. Interestingly, the abundance of Prevotella spp. decreased, while that of *Anaerobacter* spp. increased, with the age of the animal.

In contrast to the human GIT, the population of bifidobacteria present in the GIT of pigs is considerably lower, amounting to less than 1% of total bacteria or being even undetectable (22). This has been confirmed in intestinal samples (stomach, small intestine, hindgut) of piglets collected at the age of 6 h to 20 d after birth; the results of this study are tabulated in Table 2 (67).

Table 2: Presentation of the five most abundant bacteria families in piglets (*n* 6)* (adapted from Petri *et al*. 2010 (67))

	Percentages of gene sequences in sample (%)		
Family	6 h	Day 20	Average 6 h-20 days
Lactobacillaceae	1.5	44.6	23.3
Clostridiaceae	33.8	1.4	17.1
Streptococcaceae	1.5	5.4	15.4
Enterobacteriaceae	25.0	0.0	6.0
Moraxellaceae	8.8	2.7	3.5

*Data for libraries prepared from three gastrointestinal tract locations (stomach, small intestine and hindgut) of piglets (6 h-20 days); digesta collection at 6 h, 12 h, day 1, 2, 3, 5, 10, 20.

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