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Influence of High Dietary Zinc on Structure and Selected Functional Aspects of Intestinal Microbial Communities in Piglets

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Content

List of tables ..... **iii**
List of figures ..... **iv**
List of abbreviations ..... **v**
Chapter 1. General introduction **1**
Chapter 2. Literature review **3**
2.1 The pig intestinal microbiome 3
2.1.1 Introduction 3
2.1.2 Microbial gut succession 3
2.1.3 Weaning 4
2.1.4 Microbial composition in adult animals 5
2.2 Functional aspects of pig gut microbiota 6
2.2.1 Introduction 6
2.2.2 Effect of bacteria on gut development 7
2.2.3 Bacteria - host interaction 7
2.3 Zinc oxide as feed additive 13
2.3.1 Introduction 13
2.3.2 Effects of ZnO on the intestinal microbiota 14
2.4 Techniques to analyze the microbiota 16
Chapter 3. Objectives **19**
Chapter 4. The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets **21**
4.1 Abstract 22
4.2 Introduction 22
4.3 Material and Methods 24
4.4 Results 28
4.5 Discussion 37
4.6 Conclusions 41
4.7 Acknowledgements 41
4.8 Supporting material 42

4.9 Literature cited 52
Chapter 5. *Ex vivo* - growth response of porcine small intestinal bacterial communities to pharmacological doses of dietary zinc oxide 59
5.1 Abstract 60
5.2 Introduction 61
5.3 Material and methods 62
5.4 Results 65
5.5 Discussion 73
5.6 Conclusions 75
5.7 Acknowledgements 75
5.8 Author Contributions 83
5.9 Literature cited 83
Chapter 6. General discussion and conclusions 87
6.1 Discussion 87
6.2 Conclusions 98
Chapter 7. Summary/Zusammenfassung 99
Summary 99
Zusammenfassung 101
References 104
Publication List 116
Danksagung 118
Eidesstattliche Erklärung 120

List of tables

Table 4.1: Spearman correlation coefficients of protein-associated and free inorganic zinc fractions to bacterial cell numbers and their metabolites in different intestinal locations (combined for all sampling days). 1 = no signifficand correlation 36
Table 4.2: Composition of diets (as-is basis) 42
Table 4.3 Primer sequences, product length and annealing temperatures 43
Table 4.4:Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 32 days of age (n=6) 44
Table 4.5: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 39 days of age (n=6) 45
Table 4.6: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 46 days of age (n=6) 46
Table 4.7: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 53 days of age (n=6) 47
Table 4.8: Bacterial metabolites in the intestinal tract of piglets on 32 days of age [µmol g^{-1} wet weight] (n=6) 48
Table 4.9: Bacterial metabolites in the intestinal tract of piglets on 39 days of age (n=6)..... 49
Table 4.10: Bacterial metabolites in the intestinal tract of piglets on 46 days of age (n=6) (significant differences highlighted in bold numbers)..... 50
Table 4.11: Bacterial metabolites in the intestinal tract of piglets on 53 days of age (n=6)..... 51
Table 5.1: Influence of dietary zinc on the growth parameters of bacteria in the stomach and jejunum digesta samples of piglets after *ex vivo* incubation in zinc supplemented media..... 70
Table 5.2: Bacterial growth response to zinc [80 µg mL^{-1}] supplemented medium in stomach and jejunum digesta samples of piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media)..... 71
Table 5.3: Composition of diets (as-is basis) 76
Table 5.4: Primer sequences, product length and annealing temperatures..... 77
Table 5.5: Bacterial growth response to zinc [40 µg mL^{-1}and 20 µg mL^{-1}] supplemented medium in stomach and jejunum digesta samples of piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media). 78
Table 5.6: Cell numbers of bacterial groups in stomach samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide before and after 16h incubation 79
Table 5.7: Cell numbers of bacterial groups in jejunum samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide before and after 16h incubation 80
Table 5.8: Diversity indices for the enterobacterial composition after 16h incubation of stomach and jejunum samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide in media supplemented with 0 or 80 µg mL^{-1} ZnO..... 81

List of figures

Figure 2.1: Current molecular methods to analyze complex microbial ecosystems [179] 18
Figure 3.1: Interaction between microbiota and host, topic of the SFB 852 19
Figure 4.1: Heatmap compilation of bacterial cell numbers in the intestine of piglets fed low (L) or high (H) concentrations of dietary zinc. (* = significantly different between low and high dietary ZnO trial groups; $p \leq 0.05$ 31
Figure 4.2: Heatmap compilation of bacterial metabolite concentrations in the intestine of piglets fed low (L) or high (H) concentrations of dietary zinc. (* = significantly different between low and high dietary ZnO trial groups; $p \leq 0.05$) 32
Figure 4.3: Ammonia concentrations in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon 33
Figure 4.4: Total lactate concentrations in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon 34
Figure 4.5: Molar ratios of acetate in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon 35
Figure 5.1: Example for the *ex vivo* growth of bacteria in stomach digesta samples of 32d old piglets fed low or high dietary zinc in non-zinc supplemented medium (black circle) or medium supplemented with 80 µg zinc mL^{-1} (white triangle) 69
Figure 5.2: Similarity analysis of DGGE profiles from stomach and jejunum samples of piglets fed low or high dietary zinc after 16 h incubation in control- (0) or zinc supplemented medium (80) (UPGMA method).. 72
Figure 5.3: Detailed display of the bacterial growth response to zinc [80 µg mL^{-1}] supplemented medium in digesta samples of 35d old piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media) 82

List of abbreviations

ADFI	average daily feed intake
ADG	average daily gain
AGP	antibiotic growth promoter
bp	base pair
BCFA	branched chain fatty acids
CDF	cation diffusion facilitators
CFU	colony forming unit
DGGE	denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
GALT	gut-associated lymphoid tissues
GI	gastrointestinal
GIT	gastrointestinal tract
LAB	lactic acid bacteria
LPS	lipopolysaccharides
OD	optical density
PCR	polymerase chain reaction
PAMP	pathogen-associated molecular patterns
ppm	parts per million
PWD	post weaning diarrhea
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal DNA
RNA	Ribonucleic acid
rpoB	β subunit of bacterial RNA polymerase
rRNA	ribosomal RNA
SCFA	short chain fatty acid
TGE	transmissible gastroenteritis
TLR	toll-like receptor
ZnO	zinc oxide
16S rRNA	ribosomal small subunit

Chapter 1. General introduction

The use of antibiotic growth promoters in pig production has raised concerns about multi-resistant pathogenic microorganisms in the digestive system of animals and humans [1]. Since 2006 the European Union passed legislation regarding a ban of the widespread use of antibiotics as feed additive. To control post-weaning disorders and enteric bacterial diseases like diarrhea in piglet nutrition the investigation of alternative treatments gained more and more importance. To enhance animal health and performance of weaned piglets, potential ways to maintain a robust indigenous gastrointestinal microbiota include the use of feed additives such as pre- and probiotics, organic acids, plant extracts as well as minerals like zinc oxide (ZnO) [2-4].

However, what is a robust microbiota? Our knowledge of the mechanisms of the complex and dynamic gut ecosystem is still limited. The role of the huge amount of microorganisms and their influence on the host gut and immune system is not fully unraveled yet. It is confirmed that gut bacteria have an important impact on animal health. They are an integral part of the intestine and interact in many different ways with the host such as providing essential products, modulating the immune system and the immunity development and reducing pathogens by forming a barrier against pathogenic bacteria [5, 6]. The composition of gut microbial communities are determined by environmental factors such as initial contact with bacteria in early life, host immune system as well as host responses to environmental stress [7, 8]. It is assumed that the vast majority of intestinal bacteria cannot be cultured and therefore most species are not studied in detail yet.

Feed additives such as ZnO are known to directly interfere with the intestinal microbiota of farm animals by shifting the microbial equilibrium. It has been shown to improve animal performance and health of weaning piglets [9].

In this sense, studies on the mode of action of ZnO on gut microbial communities are necessary to improve knowledge regarding its extent of modification of gut flora and host.

During the last decades the research methods in microbial ecology changed from traditional culture based approaches to molecular biology techniques to assess a wider array of microorganisms in very complex environments. Ubiquitous genes like the 16S ribosomal RNA (rRNA) or the subunit of the β subunit of bacterial RNA polymerase (*rpoB*) serve as

robust phylogenetic markers [10]. Diversity studies are now dominated by quantitative PCR amplification methods (qPCR), denaturing gradient gel electrophoresis (DGGE) and next-generation sequencing [11]. However, the use of molecular methods is vital to unravel the structure and function of the pig gut microbiome.

Chapter 2. Literature review

2.1 The pig intestinal microbiome

2.1.1 Introduction

The pig gastrointestinal tract (GIT) is a highly diverse ecosystem which is composed primarily of bacteria. The species number of microbes in this habitat ranges between 400 and 600 [12-14] with densities generally increasing from the proximal to the distal GIT. The microbial communities consist of many different genera and its content can contain 10^{10} to 10^{12} cells g^{-1} wet weight [12, 15, 16]. These enormous microbial populations play a profound role in nutritional, physiological and immunological processes and have a strong influence on the health and performance of the host [17]. By colonizing their host, the microbiota protects the animal from pathogens. These commensal microorganisms alter the intestinal morphology by producing beneficial or toxic components and inducing the intestinal immune responses.
However, the microorganisms in the GIT can be found in the lumen as well as in the epithelial layers, in the crypts and even within the mucus layer of the gut [18, 19]. Throughout the intestinal tract there is a different quantity and allocation of microorganisms with lower numbers in the stomach and jejunum and the highest counts in cecum and colon [19-21]. During colonization the bacterial communities must be stable and abundant over a certain time period to avoid washout and replacement by permanent reproduction and/or adhesion to the epithelial gut layer. Important factors for a successful colonization are nutrient availability and composition, nutritional and spatial competition, viscosity of the digesta, pH and the presence of gut receptors [22, 23].
This chapter focuses on the microbial colonization and composition of the porcine GIT as well as on its severe alterations during the weaning period.

2.1.2 Microbial gut succession

Although the environment of young piglets plays a significant role, the animal body has strong selection mechanisms to ensure a specific pattern of succession of microbial gut colonization. The first colonizing populations are very diverse and cover a broad spectrum of

the environmental and mother sow microbiota. After a few days this pattern changes and piglets become more individually diverse [24]. Even among differently raised animals the progression of the microbial colonization seems similar [25]. The mechanisms of selection are not clear yet, but they seem to lead to the establishment of a diverse and stable microbial community protecting the animals from pathogens. Although variations between different species as well as different animals were observed the basic principles of succession seem to comply with similar rules [16, 26-28].

Before birth the animal GIT is sterile [29]. During birth the piglet is exposed to vaginal, skin- and fecal microbes from the mother sow and the environment which initiates the colonization process [22]. In the early colonization phase the microbiota of piglets has a high similarity with the sow intestinal microbiota [24]. Especially the uptake of sow feces after birth leads to dam related community patterns in the piglets [30].

However, the first bacterial colonizers of the gut are aerobic and facultative anaerobes including *E. coli*, lactobacilli and streptococci [22, 31]. Six hours after birth the new born animals feces already harbors as many as 10^9 to 10^{10} colony forming units (CFU) of Streptococci and *E. coli* per gram wet weight. These organisms consume oxygen to provide a reduced environment for establishing an anoxic environment. After that, facultative anaerobes and strict anaerobes follow to constitute the predominant microbial communities consisting of genera as *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Lactobacillus* [22, 32, 33]. In the suckling period the microbial composition stays stable, but the bacterial density increases rapidly. The lactic acid bacteria (LAB) are well adapted to utilize the substrate from the received colostrum and sow milk and establish a low pH environment in the gut [34-36]. It was reported that within the first ten days the total number of lactobacilli in the stomach, jejunum and ileum increased 10-fold [37]. In this phase, the microbial community is mainly composed of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Eubacterium*, *Fusobacterium* and *Streptococcus* species [33].

2.1.3 Weaning

In an animal production environment, piglets are separated from their sow after three to four weeks. The animals switch from a highly digestible milk diet to solid feed with a more complex chemical composition in this period. This process is called weaning and is associated

with social, environmental and nutritional stress [38-41]. The feed and water intake as well as animal growth are decreased as a consequence. Furthermore, the gut is more vulnerable due to changes in structure and barrier functions which may lead to diarrhea and increased mortality [42, 43]. The still fragile and developing microbial composition becomes unstable again and can be colonized by pathogenic bacteria such as *E. coli* and *Salmonella* species. It was reported that in this phase Gram-positive anaerobes are reduced and Gram-negatives are increased drastically [33]. Especially the lactobacilli populations were reduced 100-fold and enterobacteria were increased 50-fold [33, 44, 45]. These microbial disruptions diminish the capacity of absorption and digestion of nutrients and can lead to diarrhea caused by growth of pathogenic bacteria. This is the most critical time for the piglet and needs highest attention in order to preserve a stabilized gut microbiota to reduce mortality [46].

2.1.4 Microbial composition in adult animals

After weaning, intestinal bacteria develop a so called “climax community” and the adult microbiome of the gut is formed over time [43, 44]. However, most bacteria in the GIT of animals cannot be cultured and therefore most data about the microbial composition is incomplete, because there are still yet-uncharacterized bacterial species. The estimates about the number and composition of different bacterial species are controversial and depend on diet, genetic predisposition, environmental factors, measuring techniques and sampling sites [16]. Recent molecular biology techniques have increased our knowledge regarding the gut microbiota, finding that more than 80% of the phylotypes had a sequence similarity below 97% in databases. The main phylotypes were affiliated with the low-G+C Gram-positive division, the *Bacteroides* and *Prevotella* group [47]. The major bacterial groups in the pig GIT include the following genera: *Bacteroides, Bifidobacterium, Butyrivibrio, Clostridium, Escherichia, Enterococcus, Eubacterium, Fusobacteri um, Lactobacillus, Peptostreptococcus, Prevotella, Ruminococcus, Selenomonas* and *Streptococcus* [1, 16, 26, 46-48].

It is said that each bacterial species covers a specific niche and different bacteria are preferentially localized to different areas in the gut. There is also a difference in colonization density in different areas: in general, they tend to increase from the proximal to distal GIT. The stomach and proximal small intestine is characterized by high acidity (pH 4-6) and rapid flow of digesta and is therefore a challenging environment for bacterial growth. These

compartments are dominated by relatively low numbers (10^7-10^9 cells g^{-1} digesta) of acid-tolerant groups, such as Lactobacilli and Streptococci [16, 19, 49, 50]. In the ileum, the pH is almost neutral and the movement of content is lower. Therefore, a greater variety of bacterial species and a higher total number can be found (10^8-10^9 cells g^{-1}). The highest number of species occurs in the cecum and colon. The hind gut is the major site for fermentation processes in swine and is also characterized by high population diversity. It contains more than 99% strict anaerobes and 10^{10}-10^{12} bacterial cells per gram digesta [1, 12, 15]. In addition to population differences there are also high variations between mucus associated microorganisms and bacteria that exist in the digesta [27, 51].

2.2 Functional aspects of pig gut microbiota

2.2.1 Introduction

The whole microbial mass consists of more than approximately 500 different species and approximately 10^{12} cells g^{-1} digesta [12]. This is more than 10-fold the amount of host cells with a total weight of estimated 2 kg in adult pigs [52, 53]. The mammalian gut microbiota provides a range of both nutritional and protective functions such as secreting products during digestion and fermentation [39], preventing the host from pathogenic organisms forming a key barrier [15], stimulating the host immune system [40] and even modulating host gene expression [15, 40, 54-56]. Therefore, the host offers stable conditions and nutrients to provide niches for a successful and permanent bacterial colonization.

However, this alleged mutualistic relationship comes at a cost for the host. Many microorganisms generate toxic products [57], may compete with the host for nutrients [1, 58] and alter the intestinal morphology by stimulating or reducing inflammatory responses as well as by producing substrates such as butyrate or polyamines [59]. This leads to a higher epithelial cell turnover and requires the increase of mucus secretion [17, 60]. All these immunological expenses require a high investment of proteins in mucus secretion and epithelial cell shedding and may cause a lower growth performance [1].

In the following chapter the functional relationship between host and microbiota will be enlightened with respect to the role of bacteria and their influence on host gut morphology, nutrition physiology and immune development.

2.2.2 Effect of bacteria on gut development

The comparison of germ-free pigs, which are housed under sterile conditions, with conventionally kept animals gives an insight in the impact of microorganisms on the gut structure, morphology and function. The changes of anatomy, physiology and biochemistry in the presence or absence of commensal bacteria could be shown in several studies [61].

One interesting fact is that the presence of bacteria seems to have an influence on the digesta motility. In germ-free animals the gut peristaltic and therefore the movement of chyme is reduced. One reason for that may lie in the absence of fermentation products. Short-chain fatty acids (SCFA) and lactic acid are mainly produced by bacteria under anaerobic conditions and these compounds are important stimulants for gut motility. [37, 56, 62-64]. Recently, SCFAs have been shown in rodents to activate motility modulating receptors GPR41 and GPR43 existing in the enteric nervous system [65].

The intestinal mass per unit length, the intestinal thickness and length is reduced in sterile animals [66] and the cecum is enlarged with a thinner mucosal layer. The lack of microbial stimuli on the immune system may cause a reduced *lamina propria* producing less plasma cells, lymphocytes and mononuclear cells [61]. Without mucus degrading microorganisms in the cecum mucus is overproduced and leads to cecum enlargement [67].

Microorganisms influence the gut also from a morphological point of view. In germ-free animals the crypt depth is shorter and the villi height is decreased leading to a lower crypt-villi ratio. Cell turnover and growth rate are diminished and lead to a thinner cell barrier in the gut of sterile animals [68].

2.2.3 Bacteria - host interaction

One major advantage of the indigenous microbiota is assumed in the establishment of a gut barrier and a colonization resistance against pathogenic microorganisms via competitive exclusion [64, 69, 70]. The host is protected by commensals from incoming pathogenic bacteria by suppressing their colonization. Studies have demonstrated that germ-free animals are more susceptible to be infected and colonized by pathogenic bacteria than conventionally housed animals [71]. Most of the specific protection mechanisms are unclear yet, but it is believed that the suppression of colonization is caused by competition for nutrients and

attachment to mucosal surfaces, secretion of antimicrobial compounds such as bacteriocins and stimulation of the immune system [55].

Host related interaction

The host plays an important role in colonization resistance with various selection mechanisms. These include barrier functions of body surfaces and mucosal epithelia of the GIT. Additionally, certain antimicrobial peptides are constitutively produced by surface epithelial cells, and an antibacterial enzyme, lysozyme, is constitutively present in excreted body fluids. Epithelial cells secrete mucus and produce digestive enzymes and electrolytes as well as immunoglobulins. The gut also regulates digesta movement and fluidity via peristalsis and protects surfaces with high epithelial turnover rates. As shown for germ-free animals, these mechanisms are stimulated by the microbiota [72]. The epithelium consists of mucus layer, goblet cells, epithelial monolayer and the *lamina propria* . This forms a protective barrier against chemical, microbiological and physical injury. The mucus layer segregates microbes from direct contact with epithelial tissues. Furthermore, the local immune network provides antibodies, cytotoxic and T helper cells as well as phagocytic cells to reduce bacterial attachment and overgrowth by both pathogenic and commensal bacteria.

Studies could show that lymphocyte and immune cell development as well as IgA production is less in germ-free animals [73, 74]. The same studies also showed that the secretory immunoglobulin system and T cell proliferation can be developed at colonized locations in the gut by introducing selected species of commensal bacteria. This may be evidence for a co-evolutionary development of host-microbiota interactions [73].

As mentioned above, the microbiota is essential for gut maturation and immune response development, because bacteria provide antigenic material that is detected by the host. Some mechanisms on how microorganisms affect the immunity of the host are known already [75]. Studies with germ-free animals for example showed the strong influence of commensal microorganisms on maturation and development of the systemic and local immunity [76]. It was found that the absence of microorganisms in the gut leads to underdevelopment of lymphoid tissues and the mediated immunity is down regulated. This can be evidenced by lower numbers of lymphocytes in the *lamina propria* and in the lower size of the Peyer's patches as well as mesenteric lymph nodes in germ-free animals [74]. It was also shown that

the production of immunoglobulins and macrophage chemotaxis as well as the phagocytotic activity was diminished [66].

It should be noted that in animal production piglets are weaned after 3 to 5 weeks and are exposed to challenging conditions involving physical and social stress. Considering these facts, the bacterial stimulus is very important for young piglets in order to adjust the immune system. Hence, the host-microbiota relationship demands special attention [43].

However, it is not clear yet how the innate immune system distinguishes between commensal bacteria and pathogens. The bacterial tolerance on the cellular recognition sites seems to be the key for understanding the mechanisms. The gut-associated lymphoid tissue (GALT) builds the main functional barrier and is influenced by microbial stimuli supporting gut-associated immunological homeostasis [77].

Microorganisms entering the gut present pathogen-associated molecular patterns (PAMP) to recognition receptors of the immune system [78]. In host-microbe interactions, monocytes and macrophages along with dendritic cells play a crucial role in the innate immune responses against PAMP. These patterns for example are peptidoglycans and lipopolysaccharides (LPS), molecular cell surface structures that are produced only by bacteria [79].

However, many different commensal bacteria survive in the host intestine without inducing inflammatory responses. This suggests that the host recognizes these commensal bacteria through sensing systems which are distinct from those that recognize pathogenic bacteria [80].

Recent studies suggest that bacteria-derived molecules mediate interactions between the host and beneficial bacteria through sensing systems that may be different from those used for pathogenic bacteria or that molecules secreted from bacteria have roles as mediators of communication between the host intestines and bacteria [80]. It is also possible that expressional abnormalities and/or genetic mutations of receptors recognize commensal bacteria leading to their tolerance [80, 81].

However, for the complete understanding of how the immune system reacts, how bacterial colonization is modulated by the host and how certain selection mechanisms tolerate indigenous microbial populations further studies are needed.

Bacteria related interaction

Another aspect which determines the colonization pattern and success is connected to the bacterial adhesion ability. Two factors are mainly involved in the bacteria-mucus attachment process. The bacteria must express adhesins on their cell surface and goblet cells in the mucosal layer must excrete recognizable glycoproteins for a successful adherence [82]. With these chemical tools the host is able to configure the bacterial colonization pattern, but some microorganisms are also able to modulate the expression of mucosal glycoproteins [83]. Preferentially adhered target sites from non-pathogenic bacteria would promote a stable commensal community development and could potentially occupy binding sites for pathogens [84]. The expression of different cell receptors in the mucus layer is affected by genetic predisposition, cell maturity, age and diet [85]. According to these factors, the susceptibility of different colonization patterns can differ profoundly [51, 86]. Recent studies suggest that specific bacterial genera are able to actively modulate the expression of mucosal glycoproteins by changing their chemical composition in order to occupy a distinctive niche [82]. It is assumed that there is a chemical communication between microbiota and host mucus layer via biochemical signaling which leads to the alteration of the habitat [56]. Furthermore, it is suggested that mucosal carbohydrates may be used as an energy source for specific bacterial groups producing endo- and exoglycosidases to degrade oligosaccharides giving them an advantage in competing for the spatial distribution in the gut ecosystem to create new specific adhesion sites and to open new ecological niches [87, 88].

The interaction between indigenous bacteria and the intestinal mucosa can be either specific via recognition of glycoproteins or unspecific by non-specific adherence to the mucosa induced by chemotaxis and bacterial motility [89]. However, for microorganisms it is crucial to develop surface proteins for a successful attachment. Outer membrane proteins and fimbriae or pili are major parts of the surface of Gram-negative bacteria like the *Enterobacteriaceae.* Gram-positive bacteria such as lactobacilli, bifidobacteria, streptococci and staphylococci present non-protein type adhesins based on polysaccharides or lipoteichoic acids [90-92].

Another important benefit for the host is the supportive digestion and production of nutrients including beneficial compounds like certain SCFAs and B and K vitamins by the microbiota [37, 93, 94]. But also lipid utilization and mineral uptake are influenced [95, 96].

Many indigestible compounds like dietary fiber are degraded by bacterial enzymes like cellulases, hemicellulases, pectinases and xylanases [58, 97]. The microbial fermentative activities differ according to the site in the gut. In the upper intestinal tract the digestion of carbohydrates, amino acids and lipids takes place by the host and lactic acid is the main organic acid produced by bacteria. In pigs, especially the cecum, but also the colon serves as fermentation chambers. Here, undigested carbohydrates are fermented and mainly SCFA's are produced [58].

Starch, that escapes digestion in the small intestine as well as indigestible polysaccharides are the main fermentation substrates in the hind gut [58]. The fermentation of polysaccharides, oligosaccharides and proteins, peptides and glycoprotein compounds from strict anaerobes in the cecum and colon leads to high amounts of SCFAs of up to 180 mM [98] as well as lactic acid and gases like methane or carbon dioxide. The main SCFAs that result from carbohydrate and amino acids are acetate, propionate and butyrate. The typical ratio of SCFAs is about 60 acetate : 20 propionate : 20 butyrate in the pig colon depending on factors like type of chemical structure of polysaccharides, substrate availability and the microbial community composition [99, 100]. SCFAs can be absorbed from the gut lumen and act as substrates for tissues (acetate) and liver (propionate). Butyrate is a direct energy source for the colonic cells and essential for large bowel health and disease prevention [101]. Among the health benefits observed with butyrate are the prevention and inhibition of colon carcinogenesis, protection against mucosal oxidative stress, and strengthening of the colonic defense barrier and also anti-inflammatory properties [102]. SCFAs are metabolized by colonocytes via ß-oxidation and supply the body with energy [103]. Furthermore, propionate has the potential to reduce cholesterol concentrations in blood [104, 105]. In general SCFAs can also reduce diarrhea by stimulating the reabsorption of water and salts [106].

Other fermentation products such as lactate, ethanol and protons are important intermediates in the general fermentation process. These intermediates are metabolized to SCFAs by cross-feeding species in the ecosystem [107]. The most important bacterial genera involved in carbohydrate fermentation processes are *Clostridium, Bacteroides-Prevotella* , *Butyrivibrio Fibrobacter*, and *Lactobacillus* [36].

Proteolytic bacteria are able to both take up amino acids to incorporate them into bacterial protein or use them as an energy source in anaerobic conditions. Protein fermentation shows a more diverse metabolite profile compared to the carbohydrate fermentation mentioned above.

Microorganisms are able to use nitrogen from residual dietary protein, from secreted host enzymes, from mucus and even from segregated epithelial cells [108]. Proteolytic fermentation and their respective metabolites can be biochemically assigned to decarboxylation under more acidic conditions, deamination under neutral to alkaline conditions and use of sulfur-containing amino acids [109].

Catabolic fermentation of amino acids is scarce in the small intestine and occurs mainly in the hind gut, when carbon sources are depleted and under favorable pH conditions [110]. The main biochemical mechanism of protein fermentation in the hind gut leads to the production of SCFAs and ammonia via deamination. The carbon skeleton is converted to acetate, propionate, butyrate and to branched chain fatty acids (BCFAs). Isobutyrate, 2-methylbutyrate and isovalerate are formed by fermentation of branched chain amino acids valine, isoleucine und leucine [58, 111] and can be used as markers of an increased proteolytic fermentation. Many toxic metabolites can be produced during protein fermentation processes [111]. Bacterial deamination of aromatic amino acids leads to the production of phenolic compounds like phenylpropionate, phenylacetate and indole acetate from tyrosine, phenylalanine and tryptophan [111]. Amino acids which are decarboxylated under more acidic conditions are transformed to toxic biogenic amines. Hydrogen sulfide is produced by fermentation of sulfated amino acids by sulfate reducing bacteria [111].

All these toxic compounds may have an impact on pig health and performance. Evidence exists that high concentrations of ammonia increase the epithelial cell turnover causing growth depression in humans [95]. Toxic amines like histamine and cadaverine are under suspicion to increase intestinal peristalsis and cause diarrhea [112, 113]. The production of phenols and indols can also lead to reduced growth performance [114].

Also, dietary fat digestibility can be influenced by the microbiota. For proper lipid absorption the fat must be emulsified. This is carried out by bile acids. Studies showed that some bacterial groups decrease the fat digestibility by deconjugation of bile acids which may lead to a decreased fat absorption in younger animals. [115]. It has been found that *Enterococcus faecalis* and *Lactobacillus* spp. as well as *Bacillus cereus, Bacteroides* spp*., Eubacterium* spp*.,* and *Clostridium* spp. are capable of deconjugating bile acids [116]. However, there is a large number of intestinal bacteria containing enzymes to deconjugate bile [117].

2.3 Zinc oxide as feed additive

2.3.1 Introduction

For more than 20 years the addition of pharmacological doses (2000 to 3000 ppm) of zinc as ZnO to nursery diets is a common practice in pig production, especially in Asia and the Americas [118]. Previous research has demonstrated positive effects of ZnO on growth performance and health in postweaning pigs [44, 119-121]. Stanger et al. [122] could show that even challenged pigs with a gastrointestinal disease like transmissible gastroenteritis (TGE) could improve gut health and growth performance when fed 3000 ppm ZnO. As an essential element for growing pigs the recommended concentration of zinc is 50-100 mg per kg feed dry matter (National Research Council, USA). Zinc deficiency in animals is mainly manifested by growth retardation, anorexia and parakeratosis [123]. In high doses zinc can be toxic to the animals and to the environment [44]. For example, accumulation in the soil may result in reduced plant growth [124, 125]. However, the tolerable level of dietary zinc depends on the respective salt of the element. Supplied as zinc carbonate, for example the dietary addition of 2000 ppm in weaning piglets for one month produced symptoms of zinc toxicosis manifested by depressed feed intake and performance [126]. Whereas the same levels of zinc administered as ZnO did not implicate signs of toxicity [121, 127]. The reason for this can be found in the solubility of different zinc sources, as ZnO has a very low solubility at neutral pH. A high variation in the uptake of zinc from the GIT may also occur caused by possible interactions between zinc and other elements like copper, iron or calcium [121, 128, 129], but also organic sources like phytate [130, 131]. Zinc is a component of about 300 different enzyme systems [132, 133]. Various modes of actions were observed regarding the physiological effect of Zn like the stimulation of metallothionein production which is involved in maintaining Zn homeostasis [134], nutrient absorption and improvement of the intestinal morphology [135, 136]. It also appears to be necessary for porcine epithelial cell differentiation as well as for the promotion of wound healing processes [137]. Consequently, in physiological concentrations, zinc also may have a special role in resistance to infections as epithelial cells constitute the first barrier against microbial invasion.

On the other hand, many studies could show that ZnO fed in pharmacological concentrations has a preventive effect on gastrointestinal bacterial infections and diarrhea [118, 119, 121, 138, 139]

However, only few studies are available on the influence of pharmacological doses of ZnO fed to weaned piglets as well as on the effects of longer duration of dietary zinc administration on the intestinal microbiota. As the primary mode of action of pharmacological doses of ZnO is attributed to its bacteriostatic effect, more attention to a better understanding of the mechanistic aspects and function of high doses of dietary ZnO in weaning piglets is needed.

2.3.2 Effects of ZnO on the intestinal microbiota

Recent studies indicated that elevated levels of dietary zinc during the postweaning phase prevent the development of a physiological zinc deficiency [118, 140, 141] and affect the gastrointestinal microbiota [142, 143]. Zinc is also an essential trace element for prokaryotic cellular functions, acting as component of a range of enzymes such as alkaline phosphatase or terminal oxidases [139, 142, 144], but in higher doses it can act bactericidal [143].

High doses of dietary ZnO have been shown to support a large diversity of coliforms in weaned piglets. Lactobacilli colony counts were reduced, but coliform colony counts increased [145]. Also, during the post weaning period ZnO may reduce fecal counts of lactobacilli and enterococci, but only temporarily [44]. A clear decrease in the total number of anaerobes and lactobacilli and an increase in the number of coliforms in ileum samples could be shown in animals fed a high ZnO diet [142]. In a challenge study with postweaning piglets, an increased shedding of the inoculated pathogenic *Escherichia coli* strain was reported [146]. Studies could also show reduced colony counts of anaerobic and lactic acid bacteria, but no effect on *E. coli* [143]. Another feeding trial with ZnO in piglets could show a significantly increased abundance of ileal *Weissella* spp., *Leuconostoc* spp., and *Streptococcus* spp. and a reduction of *Sarcina* spp. and *Neisseria* spp. and an increase of all Gram-negative facultative anaerobic genera [147]. It could be shown that increasing ZnO levels in the diet of weaned piglets led to an increase in enterobacteria and a decrease of clostridial cluster XIVa [148]. Another study [149] showed in a feeding trial using 2,500 mg ZnO·kg feed^{-1} that species of the *Enterobacteriaceae* increased their abundance and diversity. Also, bacterial diversity indices were increased and led to an increase of less prominent species and thus had a major impact on the bacterial composition and species diversity in piglets. It could also been shown in an *ex vivo* trial that different sources of ZnO have a different mode of action on intestinal

bacteria in small intestinal samples, but generally a growth depressing effect was found [150]. Furthermore, *in vitro* studies with a broad range of intestinal reference strains for the pig intestinal tract could show that the reaction of intestinal bacteria against ZnO is species specific and that the antibacterial effect of zinc cannot be assigned to specific bacterial groups [151].

ZnO is highly insoluble in water and becomes more soluble under more acidic conditions. Administered as feed additive ZnO has to pass the stomach with its low pH environment. This increases the solubility of Zn leading to high amounts of Zn^{2+} ions (54% at 164 ppm ZnO kg^{-1} diet) [152]. The free ions of heavy metals are in general thought to have an antibacterial effect [153] and free Zn^{2+} ions may thus act bactericidal reaching the small intestine. However, it is unknown if ZnO itself has an effect on bacterial growth. Research on ZnO nanoparticles indicates that surface effects like disruption of the cell membrane and oxidative stress induced by the molecule may also be responsible [154, 155]. There are studies which suggest that zinc is a reducing factor for the virulence in pathogenic microbes. Zinc can kill or inhibit strains of *Staphylococcus aureus* [156]. Endotoxins from *Salmonella* and haemolysins from *Aeromonas* were less effective when inorganic zinc salts were present in the system [157]. Experiments on cultured cells suggest that ZnO may reduce adhesion from pathogens to intestinal epithelial cells [158].

However, high concentrations of zinc are strongly inhibitory for prokaryotes, affecting many important functions. Zinc intoxication is known to inhibit the respiratory electron transport systems of bacteria [159-161], although the toxicity of zinc is quite low compared to other heavy metals like Cd, Cu, Ni, Co [162].

Nevertheless, the mode of action of ZnO in complex environments is yet not fully understood. Prokaryotic cells developed diverse resistance mechanisms to avoid Zn intoxication [163]. These mechanisms are essential for understanding the principles of homeostatic control and maintaining the required amount of the metal and managing its excess. As mentioned above, zinc is connected with a number of processes essential for growth and metabolism, but at higher concentrations it acts toxic. Considering that both, over-expression and under-expression of resistance factors can be detrimental to the cell, it is likely that the bacterial cell is exposed to a selective pressure for the expression of influx and efflux transporters to maintain a metal homeostasis [164]. There are different strategies of resistance to toxic levels of zinc: bacterial cells can build a permeability barrier or actively export the metal from the

cell [162]. Other strategies are to produce intra- or extracellular binding proteins or other ligands to prevent it from damaging cellular targets or transform and detoxify the metal via chemical compounds [163, 165, 166]. For example, in *E. coli* three types of transporters are known: cation diffusion facilitators (CDF), protein *ZitB* and a *P-type ATPase- ZntA* [167].
Further studies are needed to comprehend the zinc resistance mechanisms in prokaryotic cells and they must be confirmed by *in vivo* trials. Studies on the bacterial transport of zinc can lead to a better understanding of the influence of ZnO as feed additive on bacterial populations in the porcine gut.

2.4 Techniques to analyze the microbiota

In order to correlate animal performance and animal health to modifications of the microbiota, appropriate tools to measure and evaluate the microbial ecology of GIT ecosystems are necessary. The first attempts to study the microbiota in chicken were done already in the 19th century [168], comprehensive surveys of the intestinal microbiota have been ongoing for more than 30 years [169]. Substantial efforts were put into characterizing the intestinal microbiota of pigs by using microbiological methods based on culturing and phenotypic analysis by selective plating combined with biochemical and morphological assays [26, 170-172].
However, plating techniques place a growth selection on bacteria. Therefore, classical microbiological methods are inadequate to accurately characterize and measure the microbiota, considering that approximately 40% to 90% of the species in the GIT are not cultivable [13, 28]. The unknown growth requirements, the selectivity of culturing media and the complexity of environmental conditions make it impossible to gather information about population dynamics and community interactions [13, 14]. Nevertheless, these studies could show that the majority of the cultivable bacteria in the pig intestine are Gram-positive, strict anaerobic bacteria from genera including *Eubacterium*, *Clostridium*, *Streptococcus*, *Lactobacillus*, and *Peptostreptococcus*, while the dominant Gram-negatives include *Bacteroides* and *Enterobacteriaceae* [172].
In the last 20 years a variety of molecular techniques has been developed that allows for a much more extensive and detailed evaluation of the microbiota in natural systems (Figure 1) [13, 16, 27, 28, 34, 36, 47, 55]. The key technique is based on the phylogenetic analysis of

DNA sequences obtained directly from samples by PCR amplification, cloning, and sequencing. Microbial community structures can be evaluated using for example 16S ribosomal DNA (rDNA) or RNA polymerase (*rpo*B) gene sequence information, using methods involving denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR), although all these procedures may be biased as well [173-176].

16S rRNA and *rpo*B genes are highly conserved in all bacteria and thus quite suitable to serve as a molecular marker [177]. Oligonucleotide sequences that recognize the conserved regions can be used as universal primers to all bacteria, whereas oligonucleotide sequences fitting to variable regions can be group specific, either to the genus, species, or even to a specific strain [178]. The use of gene sequences as molecular marker has become a standard method to identify and classify different bacterial species [13, 70]. All these molecular techniques have been used to assess the gastrointestinal microbiota in pigs and have significantly advanced the understanding of intestinal microbial ecology [13, 179]. However, the microbiota in the GIT is highly complex with a vast fraction of species not being described previously. Furthermore, a combination of classic and molecular techniques allows scientists to evaluate dietary effects on the gut microbiota.

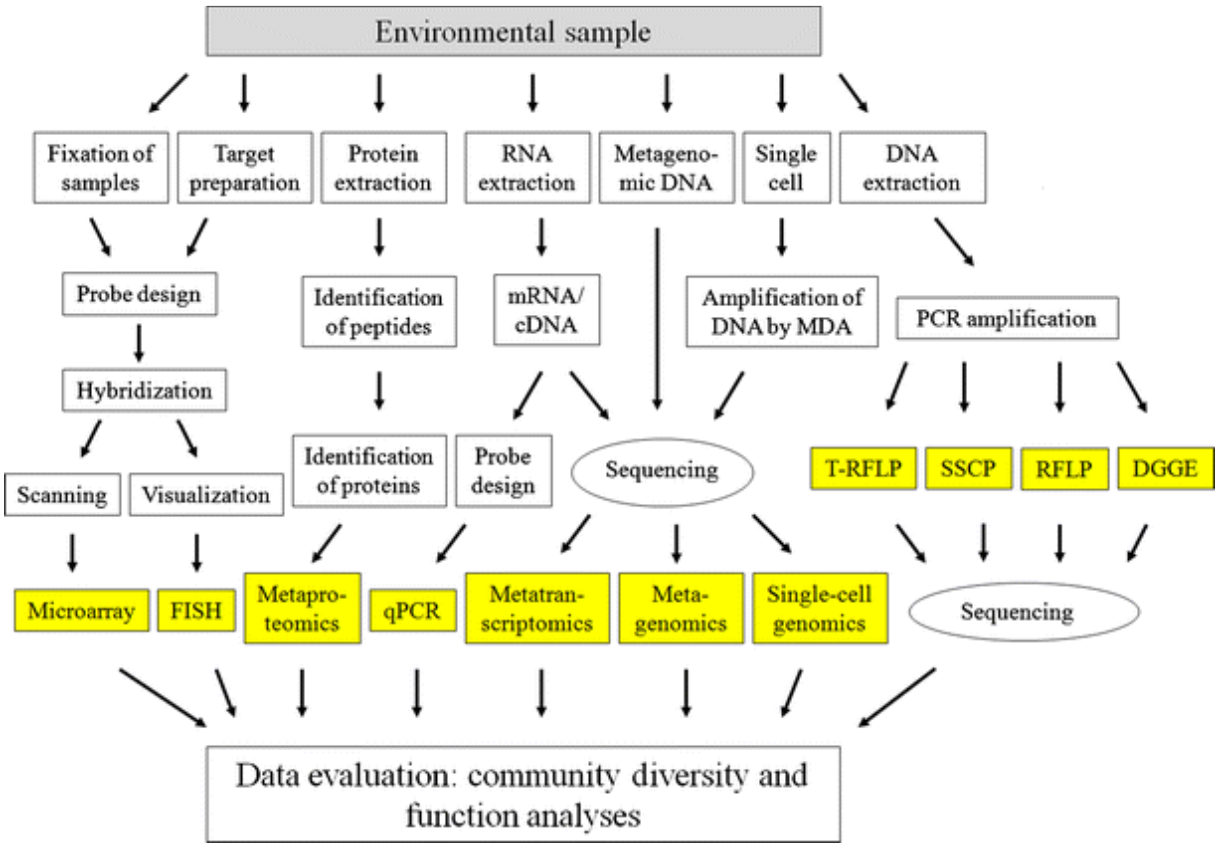

Figure 2.1: Current molecular methods to analyze complex microbial ecosystems [179]

Chapter 3. Objectives

The main objective of this thesis was to improve our knowledge of the gut microbiome of weaning piglets under the influence of dietary ZnO administered in pharmacological doses with special emphasis on lactic acid bacterial communities in the small intestine. To accomplish these aims the thesis was part of a project (A1) which was embedded in a collaborative research center (SFB 852) "Nutrition and intestinal microbiota – host interaction in the pig" representing a multidisciplinary and integrative scientific consortium focusing on a comprehensive and holistic pig model (Figure 3.1).

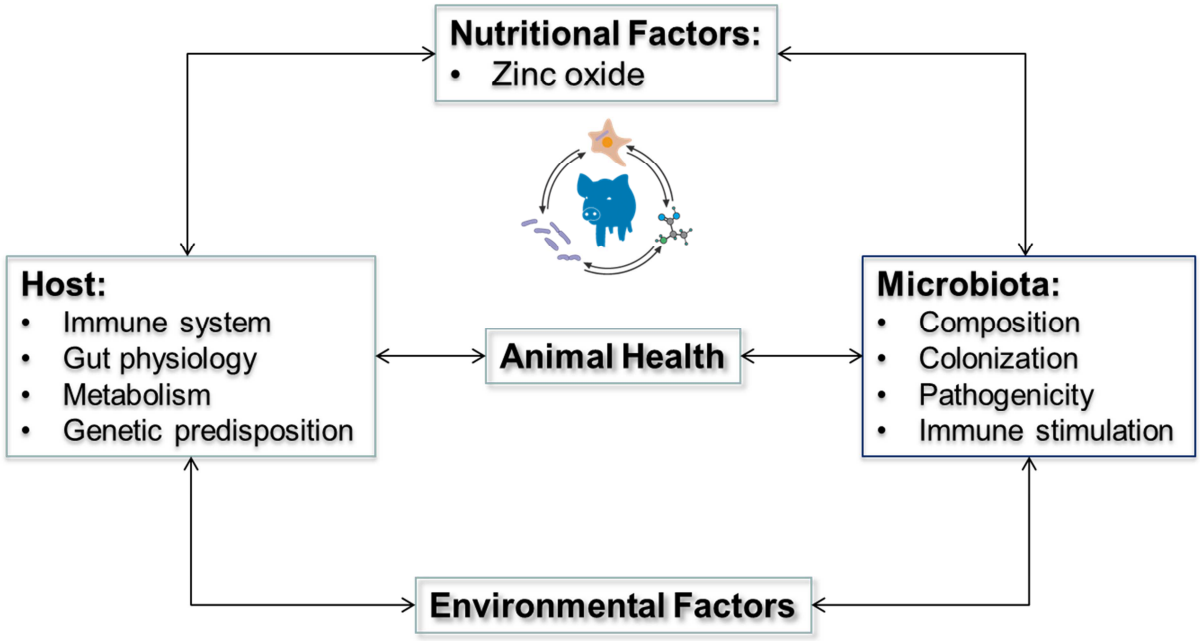

Figure 3.1: Interaction between microbiota and host, topic of the SFB 852

An important issue concerning animal nutrition is the understanding of mechanisms and specific functions of the intestinal tract. It is therefore essential to study the microbiota in the intestinal segments that are responsible for digestion, i.e. stomach and small intestine. Also, the weaning process of piglets brings many sudden changes caused by environmental and physical factors. These changes can lead to serious imbalance in the intestinal microbial community. Therefore, in an intensified pig production system it is also necessary to stabilize microbial communities after weaning to improve gut health and to reduce the risk of pathogenic infections. In this project it was the aim to assess the microbial communities in a quantitative and qualitative way. It shall allow insights into bacterial structures and changes of

the microbiota. Zinc is often used in pharmacological doses in weaned piglets to alleviate the problems mentioned above. Therefore, a zinc trial was conducted to evaluate the effect of three different Zn concentrations (57 mg kg^{-1}, 164 mg kg^{-1} and 2,425 mg kg^{-1}), provided by ZnO. Our hypotheses regarding the ZnO application are as follows:

- ZnO acts directly by reducing the bacterial populations
- ZnO has a species related influence within the microbiome
- ZnO oxide leads to interactions which modify the total bacterial community

To test the hypotheses different approaches were considered:

- Different molecular techniques were evaluated to develop a working protocol to investigate the porcine intestinal microbiota. Different DNA extraction methods as well as PCR, qPCR and DGGE were validated.
- Due to the high costs of deep sequencing analysis of the bacterial diversity denaturing gradient gel electrophoresis (DGGE) was employed as a fingerprint method to monitor the qualitative structure in the samples from the feeding trials. The single copy gene *rpoB* was used to assess qualitative modifications of the bacterial structure in the intestine of the piglets.
- Quantitative real-time PCR assays were used to monitor selected bacterial groups and species.
- *Ex vivo* conditions were set up to investigate growth and adaptive mechanisms of bacteria and analyzed by molecular methods.

The results of the thesis were obtained and evaluated with the animal trial mentioned above. The results are summarized in the manuscripts of the following chapters (**Chapter 4 and Chapter 5**).

Chapter 4. The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets

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4.1 Abstract

Weaned piglets were fed diets containing 57 (low) or 2425 (high) mg kg-1 analytical grade ZnO for a period of five weeks. Intestinal contents were sampled in weekly intervals and analyzed for bacterial cell numbers and main bacterial metabolites.

The most severe effects of high dietary zinc were observed one week after weaning in the stomach and small intestine. Pronounced reductions were observed for *Enterobacteriaceae* and the *Escherichia* group as well as for *Lactobacillus* spp. and for three of five studied *Lactobacillus* species. The impact of high dietary zinc diminished for enterobacteria with increasing age, but was permanent for *Lactobacillus* species. *Bifidobacteria*, enterococci, streptococci, *Weissella* spp. and *Leuconostoc* spp. as well as the *Bacteroides-Prevotella-Porphyromonas* group were not influenced by high dietary zinc throughout the trial.

High dietary zinc reduced bacterial metabolite concentrations and increased molar acetate ratios at the expense of propionate in the proximal intestine, but differences diminished in older animals. Lower lactate concentrations were observed in the high dietary zinc group throughout the feeding trial.

This study has shown that the application of dietary zinc at high concentrations leads to transient and lasting effects during the development of the intestinal microbiota, affecting composition as well as metabolic activity.

4.2 Introduction

The intestinal tract of young mammals is a dynamic entity within the animal and many different genetic, physiological and environmental factors govern its development into maturity (Mackie et al., 1999). Generally, the stomach functions as pre-digestive organ, as low pH, mixing of solids and liquids and peptidolytic enzymes prepare the ingested feed for digestion. In the small intestine, nutrients are hydrolyzed by digestive enzymes and transported across the epithelium. Finally, the large intestine acts as fermentation chamber, in which indigestible feed components are utilized by strict anaerobic bacteria to yield short chain fatty acids, which can be resorbed and used by the host. Birth and weaning are the most severe influences that lead to direct modification of the mammalian intestine, as nutrient

supply and contact with the environment trigger a multitude of physiological and immunological responses (Lallès et al., 2007).

An integral part of this complex and dynamic ecosystem is the intestinal microbiota, which is inescapably associated with their host (Neish, 2009). Estimates on the total of bacterial species present in the intestine range from 500 (Eckburg et al., 2005; Steinhoff, 2005 ;) to up to 1000 (Peris-Bondia et al., 2011) different bacterial species belonging to almost all branches of the bacterial tree of life (Leser et al., 2001). They outnumber the total of host cells by a factor of 10 to 100 (Shanahan, 2002) and their physiological diversity is considered as far higher than that of their host (O'Hara & Shanahan, 2006; Qin et al., 2010). Furthermore, the production SCFAs, lactate and ammonia not only influences the microbiota itself by stimulating metabolic cross feeding (De Vuyst and Leroy, 2011; Muñoz-Tamayo et al., 2011) and regulation of the bacterial gut environment (Macfarlane and Macfarlane, 2003), but can also affect the host by delivering short chain fatty acids (SCFAs) with possibly beneficial effects on health (Fung et al., 2012; Havenaar, 2011). On the other hand, detrimental metabolites such as biogenic amines and ammonia have to be detoxified (Blachier et al., 2007).

Today, it is therefore generally accepted that intestinal bacteria play an important role during the maturation of the animal in both health and disease (Sekirov et al., 2010). The stress on animals in intensive animal husbandry is especially elevated for piglets, as stocking rates are high and early weaning leads to premature intake of solid feeds, which can also lead to disease or malnourishment (Pluske et al., 2002; Lallès et al., 2007). Several different strategies are used by animal breeders to counteract the resulting losses of animals and weight gain. The main focus is set on the reduction of microbial pathogens by the use of vaccines, antibiotics or similar antibacterial agents. Among these agents, ZnO has been in use for a long time with well documented beneficial effects in animal production (Poulsen, 1995; Hollis et al., 2005; Pettigrew, 2006). The European legislation has limited the use of zinc oxide in animal nutrition to a maximum allowed concentration of 150mg ZnO per kg feed, because of suspected environmental pollution (Jondreville et al., 2003). However, in Asia and the Americas it is standard practice to use up to 3g kg^{-1} feed in weaned piglets.

Studies on the antibacterial activity of zinc are scarce; some in vitro studies show antibacterial activity in the lower mM range (Mohamed and Abo-Amer, 2012; da Silva et al., 2011; Krieger and Rein, 1982; Liedtke and Vahjen, 2012). A recent in vitro study on the minimal

inhibitory concentration (MIC) of ZnO against a wide range of intestinal strains has shown that most bacterial phylogenetic groups contain members with low or high zinc resistance, but strict anaerobic strains generally showed more diverse MIC than lactic acid bacteria or enterobacteria (Liedtke and Vahjen, 2012).

Relative to the wide-spread use of high dietary zinc in animal nutrition, there are also surprisingly few in vivo studies available on the influence of zinc on intestinal bacteria. In a study by Hojberg et al. (2005) especially lactobacilli colony counts were reduced, but enterococci were less influenced. Furthermore, increased coliform colony counts were observed. This is in agreement with a challenge study by Mores et al. (1998) which also found increased shedding of an inoculated pathogenic *Escherichia coli* strain in post weaning piglets fed zinc oxide supplemented diets. Another study by Broom et al. (2006) also reported reduced anaerobic and lactic acid bacteria counts, but no effect on E. coli was found. Recently, sequencing studies on the effect of high dietary zinc on the microbiota in piglets have shown that the enterobacterial diversity increases due to high dietary zinc (Vahjen et al., 2011). Furthermore, changes on the genus level were observed in some bacterial groups without changing the total number of sequencing reads (Vahjen et al., 2010).

However, no studies are available that combine bacterial composition and their major metabolites in a time dependent fashion. It is known that the microbiota in piglets is subjected to drastic changes during the first weeks after weaning (Konstantinov et al., 2006) and a succession of different dominating species has been shown to occur as the animal ages. The antibacterial agent zinc should have a massive impact on the natural development of the intestinal microbiota. Therefore, this study was conducted to advance the understanding of the effects of high concentrations of dietary zinc on the intestinal microbiota in piglets.

4.3 Material and Methods

Animals and Housing

Landrace piglets were weaned at 26 ± 1 days of age with a mean body weight of 7.2 ± 1.2 kg and randomly allocated into the treatment groups balancing for gender, litter and body weight. Animals were housed in pens (n = 2 per pen) with straw bedding and ad libitum access to feed and water. The study was conducted according to the German Animal Welfare Act

(TierSchG) and approved by the local state office of occupational health and technical safety 'Landesamt für Gesundheit und Soziales, Berlin' (LaGeSo Reg. Nr. 0347/09).

Diets

Diets based on a standard starter feed mixture (wheat/ barley/ soy bean meal) were fed after weaning until 54th d of life. The composition of the basal diet is shown in Supplemental Table 1. Analytical grade ZnO (Sigma Aldrich, Taufkirchen, Germany) was added to the diets. The analyzed dietary Zn concentration in the control group (low dietary ZnO) was 57 mg kg^{-1} feed, whereas the treatment group (high dietary ZnO) contained 2,425 mg kg^{-1} feed.

Performance

Body weight and feed intake were recorded on a weekly basis and average daily gain (ADG) and average daily feed intake (ADFI) were calculated. Fecal quality was monitored using a subjective scoring system ranging from 1 (entirely liquid) via 3 (normal) to 5 (hard pellets) and scoring was performed every day after the morning meal. No antibiotics were administered before and during the experiment.

Sampling

Piglets of each experimental group were sacrificed on 32 ± 1, 39 ± 1, 46 ± 1 and 53 ± 1 d of age such that treatment groups were balanced for litter and gender (n = 8). The piglets were sedated with 20 mg kg^{-1} BW of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg kg^{-1} BW of azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) prior to euthanasia by intracardial injection of 10 mg kg^{-1} BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany). Intestinal contents were taken from the stomach, mid-jejunum, terminal ileum and colon ascendens. Samples were shock-frozen in liquid nitrogen and stored at -80 °C until further analysis.

Determination of Bacterial Cell Numbers

DNA Extraction: DNA extraction was performed with a commercial kit (Qiagen Stool kit, Qiagen, Hilden, Germany) with 200 mg digesta in triplicate according to the instructions of

the manufacture except for an increase in temperature during the to 90 °C lysis step. Purified DNA was then pooled per sample.

Realtime PCR – Assays: Primer sequences and annealing temperatures are given in Supplemental Table 2. All primers were purchased from MWG Biotech (Straubing, Germany). A Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) was used for PCR amplification and fluorescent data collection. The master mix consisted of 12.5 µL Brilliant II SYBR® Green QPCR Master Mix with Low ROX (Stratagene, Amsterdam, Netherlands), 0.5 µL of each primer (10 µM) and 10.5 µl water. One µl sample was added before PCR amplification. All amplification programs included an initial denaturation step at 95 °C for 10 min to activate the polymerase. All PCR programs featured an annealing time of 60 sec and a 60 sec extension at 72 °C.

Quantification of Fluorescent Signals: A detailed description of the quantification procedure is given by Vahjen et al. (2007). This quantification method employed extracts from a large number of reference strains inoculated in a sterile matrix and thus circumvents the bias of extraction efficiency and enables the expression of results as cell number per gram sample instead of target gene copy numbers. In short, a series of autoclaved (1h, 121 °C, 2bar) sow feces samples was spiked with overnight cultures of a wide range of bacterial species and known cell numbers (109 to 103 cells per gram wet weight). After extraction and purification with the same DNA extraction protocol (Qiagen Stool kit, see above), these extracts were used as PCR calibration samples. Results were therefore expressed as cell number per gram sample wet weight.

Determination of Bacterial Metabolites

For sample preparation, 0.5 g of digesta was diluted with 1.0 mL of ice-cold 100 mM 3-(N-morpholino) propanesulfonic acid buffer (pH 7.5), homogenized for 1 min, and incubated for 10 min on ice. Samples were then homogenized again and centrifuged at 17,000 × g at 4 °C for 10 min. The supernatant was kept on ice, until 100 µL was taken for determination of the SCFA. The rest of the supernatant was mixed with 50 µL of Carrez-I and Carrez-II solutions and subsequently used for ammonia and lactate analysis. Samples were centrifuged and the supernatants were filtered by a 0.45-µm cellulose acetate syringe filter. Analysis of SCFA was carried out by gas chromatography (Agilent Technologies 6890N with auto sampler G2614A and auto injector G2613A; Santa Clara, CA). An Agilent 19095N-123 HP-

INNOWAX polyethylene glycol column was used. Then, 100 µL of the sample supernatant was diluted with 900 µL of internal standard solution, containing 0.5 mmol L-1 of caproic acid. The standard solution contained 50 mL of 10 mmol L-1 stock solution (250 µL caproic acid, 2 g of oxalic acid dihydrate in 200 mL), 2.5 g of sodium azide and 10 g of oxalic acid dihydrate in 1,000 mL.

Ammonia was quantified using a Berthelot reaction assay. Twenty microliters of the sample supernatant was mixed with 100 µL of phenol nitroprusside and 100 µL of alkaline hypochlorite in a 96-well microtiter plate. After incubation for 10 min at room temperature, a photometric measurement was carried out at 620 nm with a Tecan microtiterplate reader (Tecan Austria GmbH, Salzburg, Austria).

Analysis of d- and l-lactate was carried out with HPLC using an Agilent 1100 system with Phenomenex Chirex 3126 (d)-penicillamine 150 × 4, 6-mm column and Phenomenex C18 4.0-L × 2.0 ID mm precolumn (Agilent Technologies). Two hundred microliters of sample supernatant were filled up to 1 mL with copper-II-sulfate solution (0.5 mmol). The column temperature was 35 °C and the UV detector wavelength was 253 nm.

Determination of Total, Free and Protein-associated Zinc

Samples were initially diluted (1:2 vol : vol) in water, homogenized for 1 h at room temperature and centrifuged at 14,200 x g for 10 min. Centrifugates were used to determine total insoluble zinc in the sample. Supernatants were withdrawn quantitatively and applied on polymeric reversed-phase sorbent columns (8B-S100-FBJ, Phenomenex, Aschaffenburg, Germany). The resulting eluents contained the total free inorganic zinc of the samples. Protein-associated zinc was determined from eluents after elution with acetonitrile/water (4:6 vol : vol) and acetonitrile/formic acid (7:3 vol : vol) and evaporation of the organic phase by vacuum centrifugation. Total zinc in the sample fractions was determined in an atomic absorption spectrometer (AAS vario 6, Analytik Jena, Germany) after total hydrolysis of sample fractions in hydrochloric acid (37%) for 90 min at 250 °C.

Graphical Presentation of the Impact of Zinc on the Development of the Intestinal Microbiota

Mean data of bacterial cell numbers and metabolite concentrations were used to construct heatmaps with the web tool “Heatmap” (http://www.hiv.lanl.gov) which uses "heatmap.2" of

the gplots package of the statistical environment R. Significant differences between trial groups were then marked with asterisks. 3D plots showing the development of bacterial metabolite concentrations along the intestinal tract were generated with the software SigmaPlot 11.0 (Systat Software, Inc., Erkrath, Germany).

Statistics

Statistical analysis was carried out with SPSS 19.0 (SPSS Inc., Illinois, USA). The Kolmogorov-Smirnov-Test was used to test normal data distribution. Normal distributed data was analyzed for significant differences using the Students t-test. Significant differences of non-normal distributed data were determined by the Kruskal-Wallis Test followed by the Mann-Whitney-U test. Differences at $P<0.05$ were considered significant.

4.4 Results

Performance of animals

All animals remained clinically healthy during the entire period and diarrhoea (fecal scores< 2.5) occurred only very occasionally with no differences between treatments. The average daily weight gain (ADG) and average daily feed intake (ADFI) were higher ($P<0.05$) in piglets fed high ZnO level during the first week as compared to the other group, but this effect almost reversed after 3 weeks with higher ADG in the low ZnO group (Martin et al., 2012).

Bacterial Composition

Means and standard deviations for all data are shown in Tables 4.3 to 4.6. Figure 4.1 displays a heatmap of all studied bacterial groups and species for all sampling days and intestinal segments. The *Bacteroides–Prevotella–Porphyromonas* group, bifidobacteria, enterococci, streptococci, *Weissella* spp. and *Leuconostoc* spp. showed no significant differences between trial groups at any sampling time or intestinal location. Similarly, high dietary zinc invoked no or only marginal differences for three clostridial clusters, except for the clostridial cluster IV in the jejunum and ileum and for the clostridial cluster I in the colon on sampling day 42. *Enterobacteriaceae* as well as the *Escherichia-Hafnia-Shigella* group were significantly reduced due to high dietary zinc on day 35, but later sampling days showed no differences. Conversely, lactobacilli and especially three of five studied Lactobacillus species responded

to high dietary ZnO with reduced cell numbers throughout the trial period, the exception being *L. johnsonii* and *L. reuteri*, which only showed significantly reduced cell numbers in the high dietary ZnO group on the 35th day of life. Generally, the most pronounced differences were observed in the small intestine (jejunum, ileum) and in the first sampling week.

Bacterial Metabolites

Means and standard deviations for all data of short chain fatty acids and their molar ratios, lactate and ammonia as well as total metabolites are shown in Tables 4.7 to 4.10. A heatmap for bacterial metabolites is shown in Figure 4.2. As expected, lactate was the main metabolite in the stomach and small intestine, while acetate and propionate were the dominant SCFA in the large intestine. Although significant differences were infrequent due to high individual differences, reduced concentrations of SCFA, ammonia and lactate were visible at almost all sampling sites and sampling days. Figure 4.3 shows that high dietary zinc reduced concentrations of ammonia especially in the jejunum and colon. However, in the 2nd to 4th week after weaning, fewer differences were observed. Total lactate was drastically reduced in the small intestine throughout the trial (Fig. 4.4). Ratios of the major short chain fatty acid acetate were generally higher in animals fed the high dietary zinc concentration throughout the trial (Fig. 4.5). Concurrently, propionate generally showed higher numeric ratios in animals fed the low dietary zinc diet, but significant differences were infrequent (see Tables 4.7-4.10). Overall, total metabolites were reduced considerably throughout the trial with often significant differences in the small intestine.

Effects of Protein Associated and Free Inorganic Zinc Fractions on Bacterial Cell Numbers and Metabolites in different intestinal sites

A spearman correlation analysis of protein-associated and free inorganic zinc fractions to bacterial cell numbers and free inorganic zinc combining all sampling days is shown in Table 5.1. While free inorganic zinc showed most interactions in the stomach and small intestine, no interactions were observed for protein associated zinc in the stomach. There were also generally less interactions between protein associated zinc and bacterial parameters than for free inorganic zinc.

As expected, most correlations were negative for free inorganic zinc, but some noteworthy exceptions were observed. Thus, combined for all sampling days, free inorganic zinc showed

positive dependencies for the *E. coli* group in the proximal intestine. The same was true for the strict anaerobic Gram-negative *Bacteroides-Prevotella-Porphyromonas* cluster as well as for *Enterococcus* spp. Also, the clostridial cluster XIVa correlated negatively with free inorganic zinc in the proximal intestine, but a positive correlation was observed in the hind gut. All studied Lactobacillus spp. strains showed only negative dependencies for the zinc fractions, but bifidobacteria seemed not influenced at all.

Surprisingly, no significant correlations were visible for short chain fatty acids except positive dependencies in the jejunum. Ammonia was negatively correlated to free inorganic zinc, but except for negative dependencies in the stomach, L-lactate only showed negative correlations to protein-associated zinc in the small and large intestine.

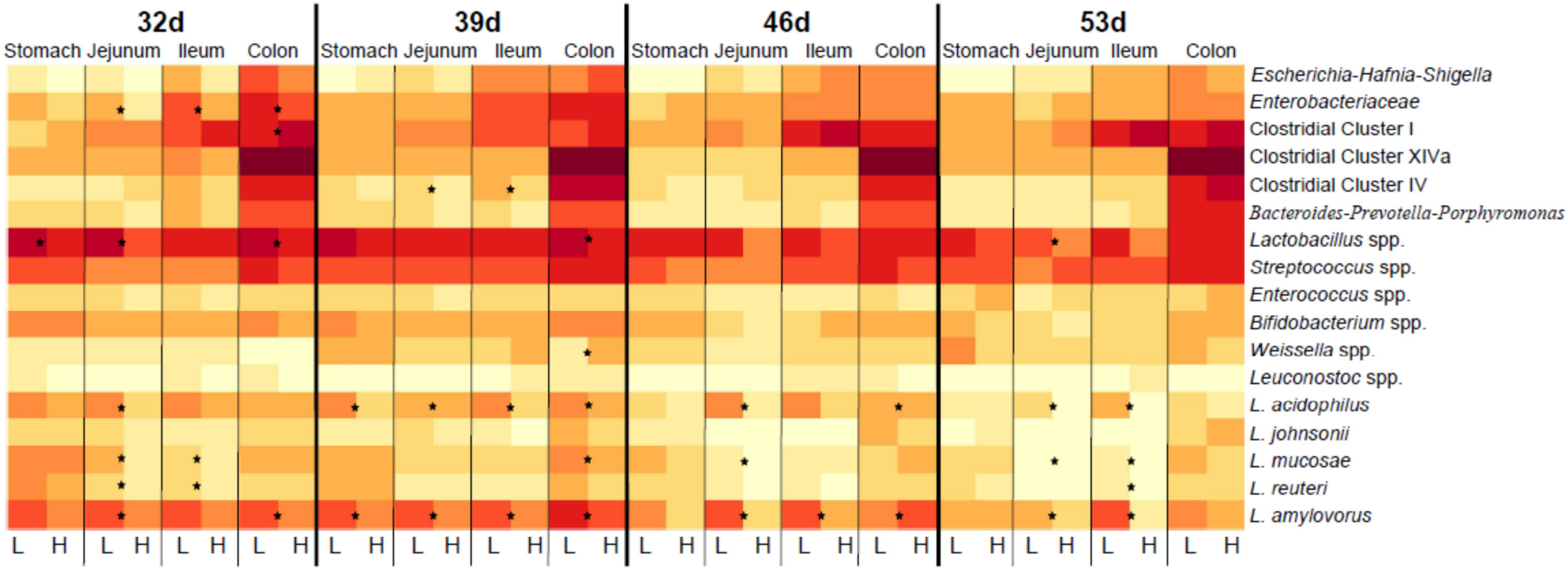
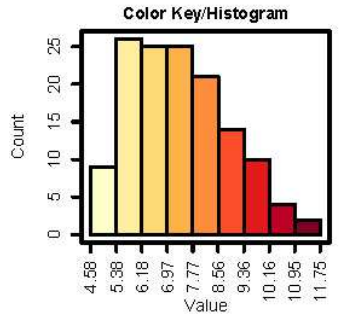


Figure 4.1: Heatmap compilation of bacterial cell numbers in the intestine of piglets fed low (L) or high (H) concentrations of dietary zinc. (* = significantly different between low and high dietary ZnO trial groups; $p \leq 0.05$



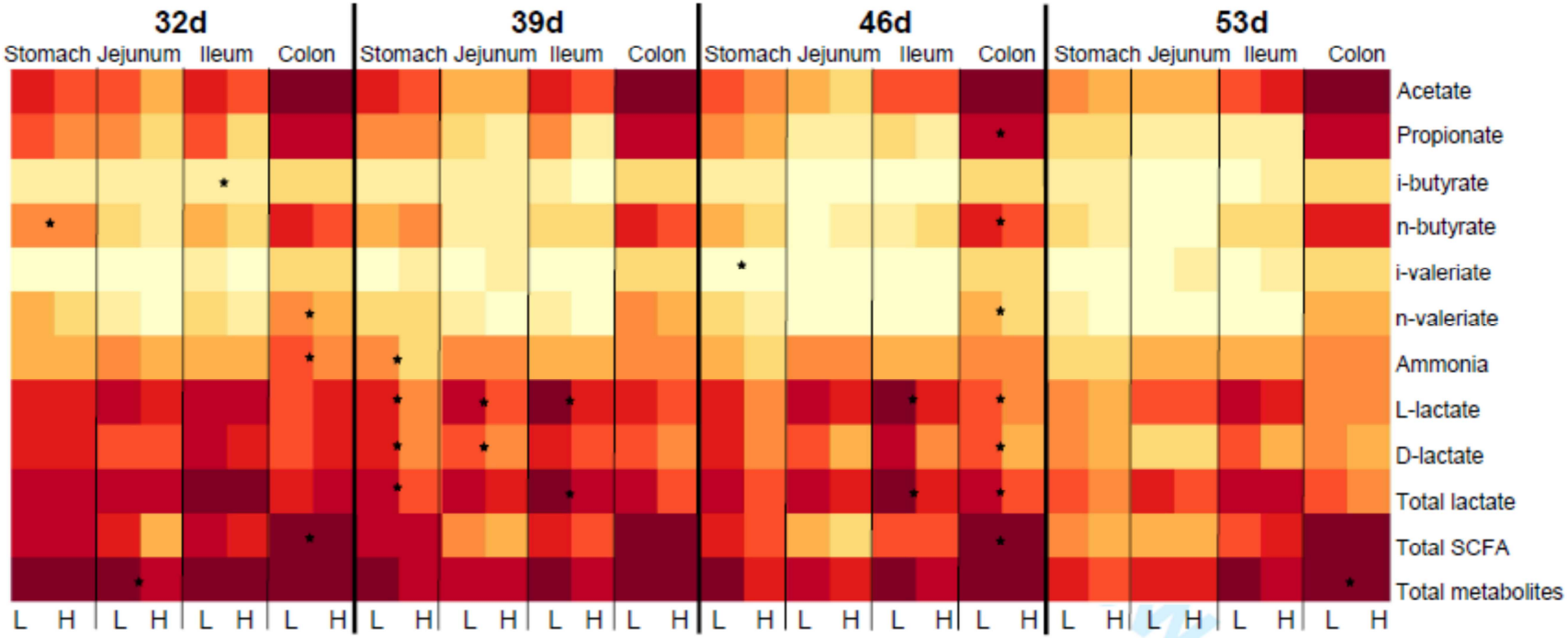
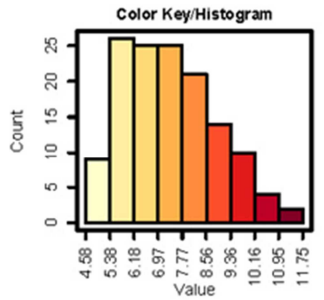


Figure 4.2: Heatmap compilation of bacterial metabolite concentrations in the intestine of piglets fed low (L) or high (H) concentrations of dietary zinc. (* = significantly different between low and high dietary ZnO trial groups; $p \leq 0.05$)



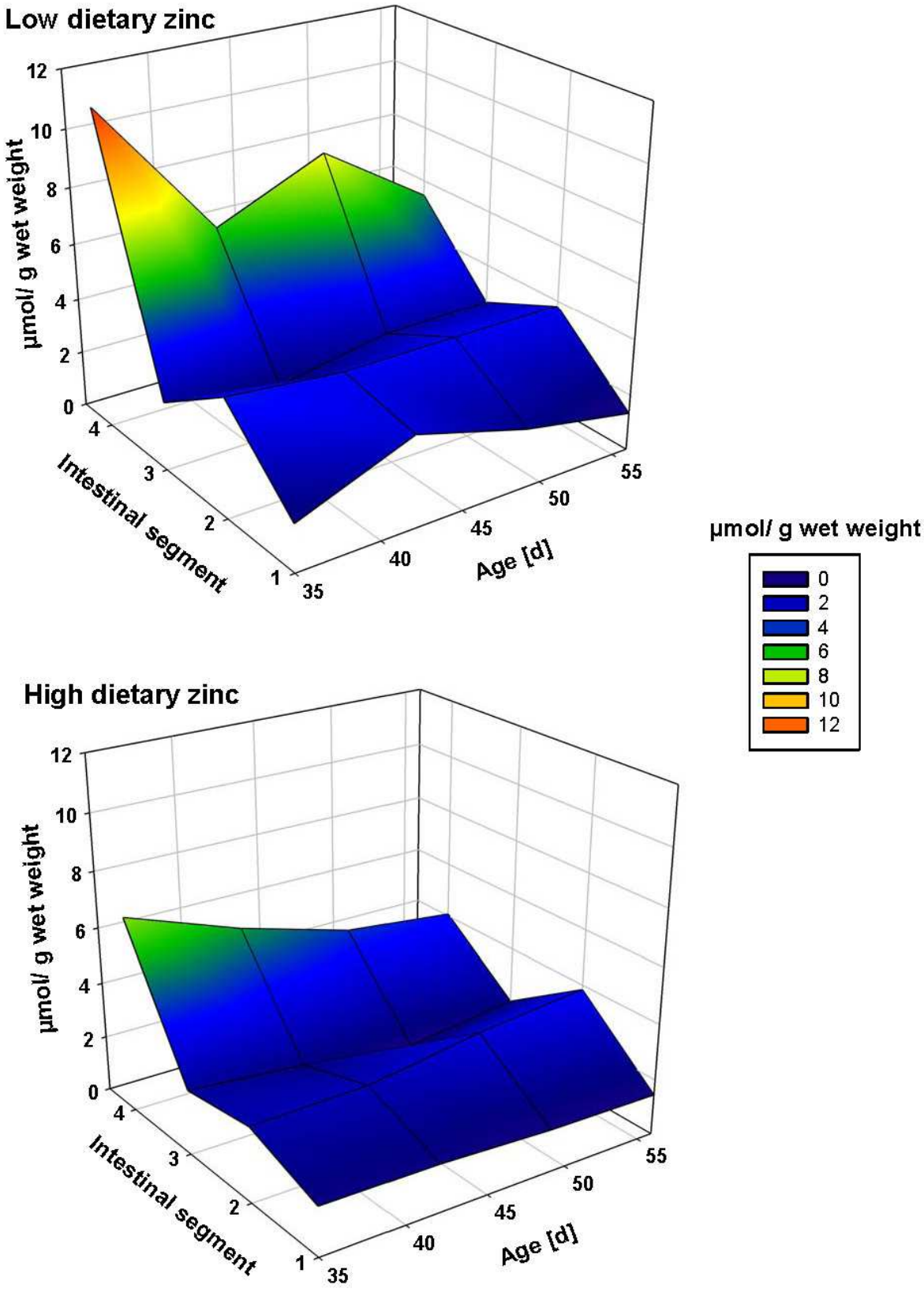


Figure 4.3: Ammonia concentrations in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon

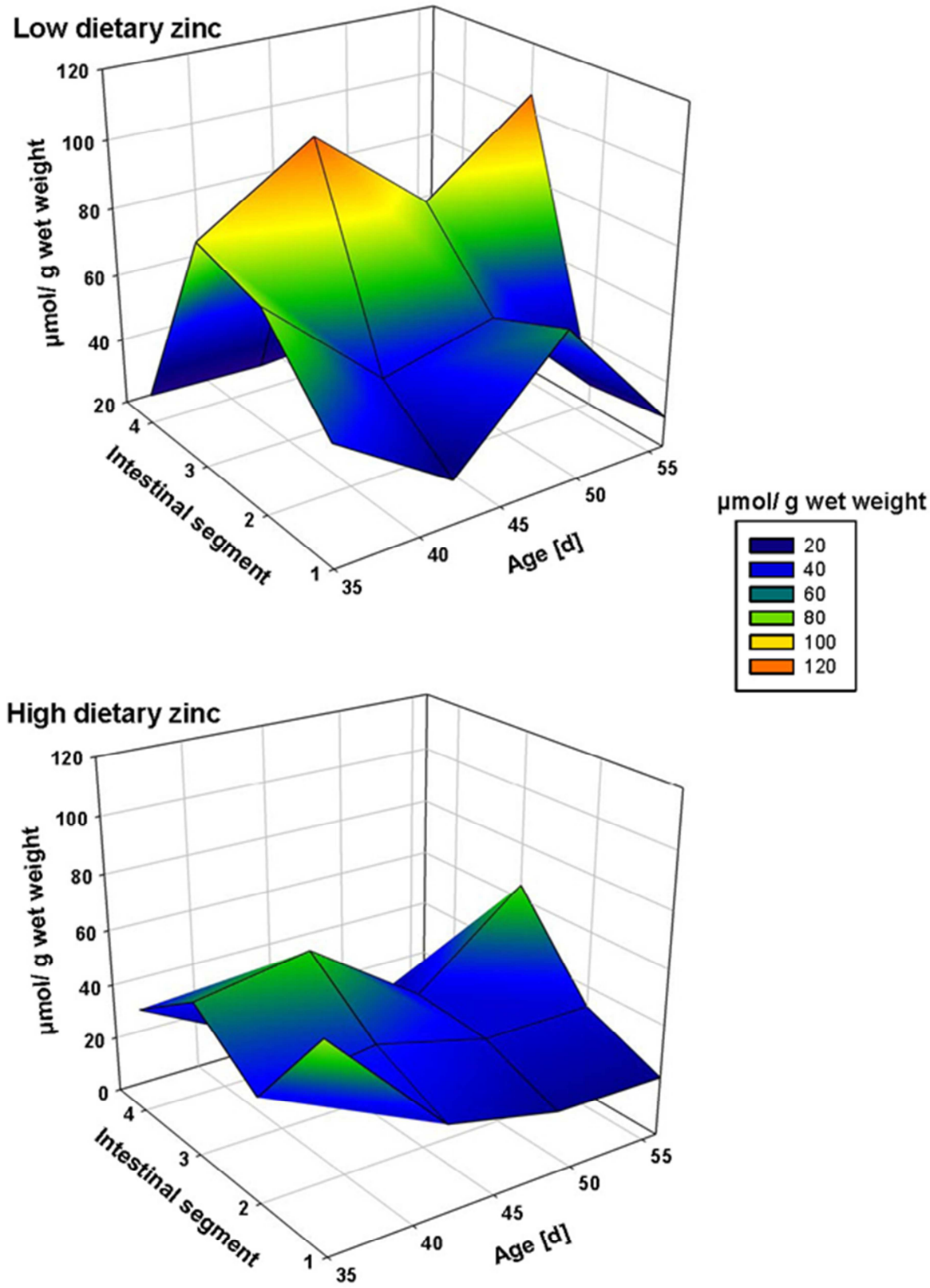


Figure 4.4: Total lactate concentrations in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon

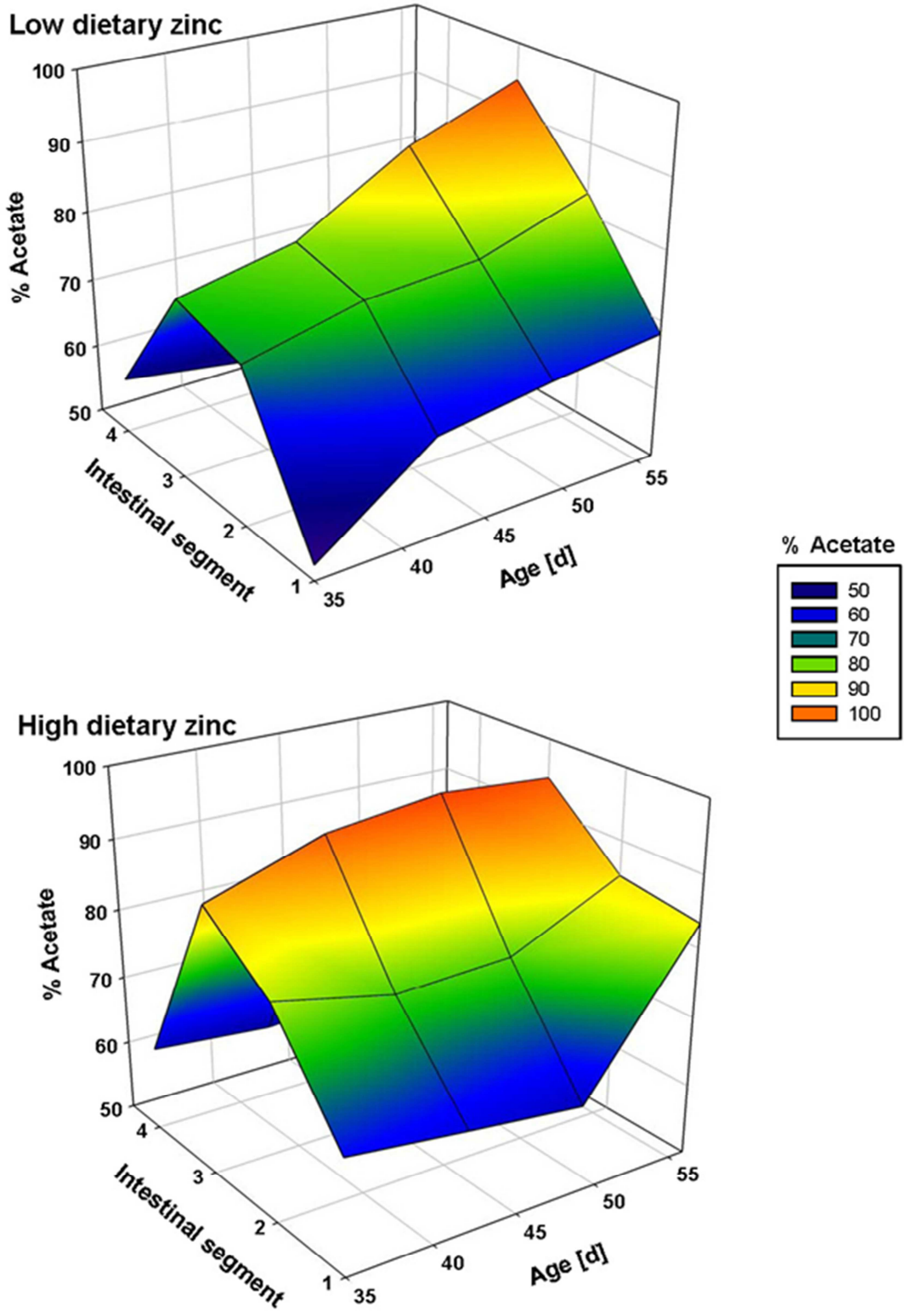


Figure 4.5: Molar ratios of acetate in the intestine of piglets fed low or high concentrations of dietary zinc [µmol/g wet weight]. Segments: 1 = stomach, 2 = mid-jejunum, 3 = terminal ileum, 4 = colon

Table 4.1: Spearman correlation coefficients of protein-associated and free inorganic zinc fractions to bacterial cell numbers and their metabolites in different intestinal locations (combined for all sampling days). 1 = no signifficand correlation

	Free inorganic zinc				Protein associated zinc			
Item	Stomach	Jejunum	Ileum	Colon	Stomach	Jejunum	Ileum	Colon
E. coli/Hafnia/Shigella	0.339	0.355	-[1]	-	-	-	-	-
Enterobacteriaceae	-0.275	-	-	-	-	-	-0.31	-
Clostridial cluster I	-0.247	-	-	-	-	-	-	-
Clostridial cluster XIVa	-0.378	-0.259	-	0.501	-	-	-	-
Clostridial cluster IV	-0.457	-0.242	-	-	-	-	-0.334	0.341
Bacteroides-Prevotella-Porphyromonas	0.296	0.62	-	0.582	-	-	-0.275	-
Lactobacillus spp.	-	-	-	-0.343	-	-0.389	-0.342	-
Streptococcus spp.	-0.455	-0.513	-	-	-	-	-	-
Enterococcus spp.	0.257	0.265	-	0.598	-	-	-	-
Bifidobacterium spp.	-	-	-	-	-	-	-	-
Weissella spp.	-0.298	-0.259	-	-0.277	-	-	-	-
Leuconostoc spp.	-	-	-	-0.343	-	-	-	-
L. acidophilus	-0.29	-0.248	-0.335	-0.382	-	-0.557	-0.512	-0.36
L. amylovorus	-0.293	-	-0.375	-0.379	-	-0.541	-0.528	-0.274
L. johnsonii	-0.326	-0.611	-0.349	-	-	-	-	-
L. mucosae	-	-	-0.325	-	-	-0.454	-0.5	-
L. reuteri	-	-	-0.337	-	-	-0.463	-0.54	-0.277
Total volatile fatty acids	-	0.269	-	-	-	-	-	-
Acetate	-	0.252	-	-	-	-	-	-
Propionate	-	0.353	-	-	-	-	-	-
i-Butyrate	-	-	-	-	-	-	-	-
n-Butyrate	-	0.395	-	-	-	-	-	-
i-Valerate	-	-	-	-	-	-	-	-
n-Valeriate	-	0.413	-	-	-	0.295	-	-
Ammonia	-0.246	-0.441	-	-0.484	-	-	-	-0.29
L-Lactate	-0.255	-	-	-	-	-0.326	-0.401	-0.287
D-Lactate	-	-	-	-	-	-	-	-0.302

4.5 Discussion

This study was conducted to describe the influence of high dietary zinc on the development of the intestinal microbiota and their metabolic activity in the intestine of weaned piglets.

The most drastic impact of high dietary zinc was the lasting reduction of three of five studied *Lactobacillus* species. Lactic acid bacteria and especially lactobacilli are dominant in the stomach and small intestine of piglets (Sghir et al., 1998; Hill et al., 2005; Pieper et al., 2008; Vahjen et al., 2010). Among the lactobacilli, *L. amylovorus* and *L. reuteri* are frequently found to dominate the *Lactobacillus* spp. populations in the small intestine (Konstantinov et al., 2004; Hojberg et al., 2005; Metzler-Zebeli et al., 2010). *L. amylovorus* was also the most prevalent species among the *Lactobacillus* spp. that were analysed in this study and one of the species most severely influenced by high dietary zinc. However, as cell numbers of total lactobacilli were much higher than the combined cell numbers of analysed species, other *Lactobacillus* spp. not analysed in this study have contributed to total lactobacilli. Nevertheless, given the enormous diversity of lactobacilli in the small intestine of piglets (Leser et al., 2001; Vahjen et al., 2011), it is probable that many other *Lactobacillus* spp. contributed to the total lactobacilli amount at lower cell numbers.

The decrease of *L. amylovorus* coincided with lower lactic acid concentrations due to high dietary zinc throughout the feeding trial. This effect seemed to be confined to lactobacilli only, as other lactic acid producing bacteria were not significantly reduced in cell numbers (bifidobacteria, enterococci, streptococci, *Weissella* spp. and *Leuconostoc* spp.).

Interestingly, *L. reuteri* and especially *L. johnsonii* were less affected by high dietary zinc. Thus, the antibacterial action of zinc in vivo is probably not intrinsic to lactobacilli per se, but may rather be species specific. This was also shown by Liedtke and Vahjen (2012), who observed differing in vitro zinc resistance among 9 Lactobacillus species of intestinal origin.

A pyrosequencing study on the bacterial core in the ileum of 40-42 d old piglets with similar high zinc concentrations also reported that Lactobacillus species were reduced, but *Weissella* spp. and *Leuconostoc* spp. significantly increased due to high dietary zinc (Vahjen et al., 2011). Those results could only in part be confirmed in this study, as ileal *Weissella* spp. and *Leuconostoc* spp. cell numbers increased only numerically on day 42 and instead of significant decreases of *L. reuteri*, significant decreases of *L. amylovorus* were observed.

Nevertheless, it seems that the niches opened by reduction of one or more bacterial species can be used in part by other species, in this case *Weissella* and *Leuconostoc* species.

The second most drastic influence of high dietary zinc was noted for enterobacteria. However, contrary to lactobacilli, the reduction in total enterobacterial cell numbers was confined to the first week after weaning. As individual enterobacterial species were not analyzed in detail, it remains unknown whether zinc sensitive species were replaced by resistant species or if enterobacterial species adapted to the high zinc conditions. However, a pyrosequencing study with 40-42d old piglets has shown that the diversity of enterobacteria increased due to high dietary zinc (Vahjen et al., 2011). Furthermore, other studies on high dietary zinc in piglets also show either no effect (Broom et al., 2006) or an increase in coliforms (Hojberg et al., 2005) as well as an increased phenotype stability of coliform isolates after weaning (Katouli et al., 1999). Therefore, it is likely that enterobacteria possess mechanisms to successfully counteract high dietary zinc concentrations. This effect may be achieved actively by more efficient heavy metal efflux systems to expel intracellular zinc (Nies, 2003; Nies and Silver, 1995) or due to gene transfer of specific heavy metal plasmids, which have been detected in both Gram-positive and Gram-negative intestinal bacteria (Chen et al., 2008; Obrien et al., 2002; Nies, 2003; Silver and Walderhaug, 1992). Less information is available on specific heavy metal plasmids of lactobacilli (Fortina et al., 1993). An enhanced colonization may also occur by passive means such as reduced bacterial competition with other bacterial groups. In this study, the reduction of the resident dominant lactobacilli as well as their main metabolite lactate may have led to a more undisturbed intestinal colonization of enterobacteria. Finally, an increased colonization potential and possibly increased diversity of enterobacteria due to high intestinal zinc concentrations may also have an impact on pathogenic *E. coli* strains. Under field conditions, *E. coli* induced diarrhea is often reduced in piglets fed high dietary zinc diets. Therefore, pathogenic *E. coli* strains may encounter a higher intra-group competition due to increased diversity of enterobacteria or their resistance to zinc and may thus be at a disadvantage to colonize the porcine small intestine.

The cell numbers of all studied clostridial cluster and the large Gram-negative *Bacteroides-Prevotella-Porphyromonas* cluster seemed to be unaffected by high dietary zinc concentrations on the whole. Again, as individual species of these bacterial groups were not analyzed, it remains unknown if a similar effect as observed for lactobacilli occurred, i.e. certain sensitive species may have been affected, while other more resistant species gained in

cell number, resulting in no visible changes overall. Both bacterial groups are strict anaerobic, fastidious and therefore attain high cell numbers predominantly in the large intestine, although they can be detected at lower cell numbers in the small intestine. The combined correlation analysis for all sampling days showed that the clostridial cluster XIVa exhibited a negative dependency to free zinc ions in the small intestine like most other bacterial groups, but a positive dependence in the hind gut. It may therefore be possible that the already dominant clostridial cluster XIVa gained an additional colonization advantage in the hind gut during the development of the microbiota. The cause for this effect may again be based on a higher zinc resistance of certain species, which would increase their cell number. On the other hand, a displacement of other bacterial groups such as lactobacilli would lead to reduced competition in the small intestine. This could be especially true for the *Bacteroides-Prevotella Porphyromonas* cluster, as positive correlations to free zinc were also found in the small intestine, where lactobacilli usually dominate.

The total concentration of bacterial metabolites was almost always lower in all intestinal segments and all sampling days. This confirms the bacteriostatic effect of pharmacological doses of dietary zinc. Interestingly, propionate concentrations were also reduced in the small intestine. Propionate is not only produced from carbohydrate fermentation, but also by lactate fermenting bacteria. Among the lactate fermenting bacteria found in the stomach and small intestine, *Veillonella* spp. (McGillivery and Cranwell, 1992; Kraatz and Taras, 2008) are known to heavily ferment lactate. However, clostridia that are present in the stomach and small intestine of young piglets as well as other strict anaerobic bacteria such as *Megasphaera elsdenii* or *Selenomonas ruminantium* also use lactate as energy source (Nagaraja and Titgemeyer, 2007; Ingham et al., 1998). The metabolism of lactate fermenting species may therefore also be limited due to reduced substrate input in the small intestine.

Total metabolites in the large intestine were also always lower in the high dietary zinc group; mainly because of a decrease of propionate concentrations (significantly so on day 49 and 56) and in part due to decreased n-butyrate concentrations, while acetate concentrations remained unchanged. As mentioned, lactate serves as substrate for propionate, but also for n-butyrate production in the intestinal tract. While the human microbiota seems to produce mainly n-butyrate from lactate (Bourriaud et al., 2005), the rumen microbiota produces more propionate from lactate (Nagaraja and Titgemeyer, 2007).

Knowledge about the composition of lactate fermenting bacteria in the large intestine of pigs is scarce, but similar species as in humans and ruminants have been described. The use of lactate as substrate in the hind gut is of course dependent on lactate production and thus, it is reasonable to conclude that a reduced lactate concentration would reduce a metabolic cross-feeding. Lower lactate concentrations could directly lead to reduced propionate concentrations and may therefore also have an impact on hind gut microbiota, which could be modified due to reduced small intestinal input of metabolites and a different bacterial species composition. A reduced lactate production would also have implications on bacterial species that rely on lactate as sole energy source (Nagaraja and Titgemeyer, 2007).

From the comparison of bacterial cell numbers and metabolites it can be concluded that high dietary zinc acted bacteriostatic on the entire microbiota, because total detected cell numbers were only marginally reduced during the first week after weaning, but total metabolite concentrations were still reduced after four weeks. As total bacterial cell numbers were not much influenced by zinc, this also indicates that the intestinal microbiota as a whole community has a high capacity to adapt by replacement of certain species by other species that are able to thrive under the given conditions.

Overall, the first week after weaning showed the highest differences between low and high dietary zinc intake. Weaning forces the animal into an extreme stress situation, because it has to adjust to a new environment, to the change from liquid mother milk to uptake of solid feed as well as to social stress. The consequences are readily visible by refusal or only marginal intake of feed during the first three to four days after weaning and therefore the intestinal physiology and immune response of the animal are deeply disturbed. As a result, the intestinal microbiota itself is imbalanced, causing diarrhoea due to opportunistic pathogens such as E. coli. As the microbiota is far from equilibrium, any further factor that takes effect on bacteria during this time must induce additional modifications in the already perturbed habitat. In this case, zinc acted as a powerful modifier, which affected certain bacterial groups (Lactobacilli/ enterobacteria). It remains unknown, if the lasting reductions of certain *Lactobacillus* spp. species are due to their inability to adapt to high zinc concentrations or if their initially slower development led to reduced colonization due to later competition from already established bacterial species.

4.6 Conclusions

The cell numbers of a range of bacterial groups indicate that pharmacological doses of dietary zinc oxide act mainly in the stomach and small intestine primarily through a reduction of certain Lactobacillus species. A reduced competition may give rise to increased colonization by other bacterial groups or species. The most drastic effects occurred during the first two weeks after weaning. Therefore, and in accordance with Broom et al. (2006) we propose that it may be sufficient to supplement diets with ZnO during this early post weaning phase. This would also reduce the environmental hazard of ZnO containing pig manures (Vellenga et al., 1992; Jondreville et al., 2003; Shi et al., 2011).

4.7 Acknowledgements

The technical support by M. Eitinger, K. Topp and A. Kriesten is gratefully acknowledged. This study was financially supported by the German Research Foundation (DFG) Grant No. SFB 852/1.

4.8 Supporting material

Table 4.2: Composition of diets (as-is basis)

Item	
Ingredients, g/kg	
Wheat	380
Barley	300
Soybean meal	232
Corn starch/ zinc oxide[1]	10
Limestone	20
Monocalcium phosphate	20
Mineral & Vitamin Premix[2]	15
Soy oil	17.5
Salt	2.0
Lysine HCl	2.5
Methionine	1.0
Calculated contents	
Dry matter, g/ kg	879
ME, MJ/kg	13.0
Crude ash, g/ kg	81
Crude protein, g/ kg	194
Crude fiber, g/ kg	36
Ether extract, g/ kg	34
Starch, g/ kg	376
Lysine, g/ kg	11.7
Methionine, g/ kg	4.0
Threonine, g/ kg	7.2
Tryptophane, g/ kg	2.4
Calcium, g/ kg	11.0
Phosphorus, g/ kg	8.0
Sodium, g/ kg	3.1
Magnesium, g/ kg	2.2
Zinc, mg/kg[3]	34
Iron, mg/kg	309
Manganese, mg/kg	81
Copper, mg/kg	18

[1] Corn starch in the basal diet was partially replaced in the diets containing 50 and 2500 mg/kg zinc with analytical grade zinc oxide (Sigma Aldrich, Taufkirchen, Germany) to adjust for the zinc level.
[2] Mineral and Vitamin Premix (Spezialfutter Neuruppin Ltd., Neuruppin, Germany), providing per kg feed: 1.95 g Na (as sodium chloride), 0.83 g Mg (as magnesium oxide), 10,500 IU Vitamin A, 1,800 IU Vitamin D3, 120 mg Vitamin E, 4.5 mg Vitamin K3, 3.75 mg Thiamine, 3.75 mg Riboflavine, 6.0 mg Pyridoxine, 30 µg Cobalamine, 37.5 Nicotinic acid, 1.5 mg Folic acid, 375 µg Biotin, 15 mg Pantothenic acid, 1200 mg Choline chloride, 75 mg Fe (as Iron-(II)-carbonate), 15 mg Cu (as Copper-(II)- sulfate), 90 mg Mn (as Manganese-(II)-oxide), 675 µg J (as Calcium-iodate), 525 mg Se (as Sodium-selenite).
[3] Analyzed concentration of zinc in the basal diet without ZnO supplementation. The other diets contained 57 and 2425 mg/kg, respectively.

Table 4.3 Primer sequences, product length and annealing temperatures

Specificity (target gene)	Primer name	Sequence	Product length[bp]	Annealing temperature [°C]	Reference
E. coli/ Hafnia/ Shigella (16S rRNA)	Entero-F Entero-R	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	340	58	1
Enterobacteriaceae (rpoB)	EntqPCR3417f EntqPCR3852r	GTBTCDCCRCGCAGRC TGCGYCTGGTRATCTA	435	58	6
Clostridial-Cluster I (16S rRNA)	CI-F1 CI-R2	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	231	63	5
Clostridial-Cluster XIVa (16S rRNA)	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	440	60	4
Clostridial-Cluster IV (16S rRNA)	sg-Clept-F sg-Clept-R	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	239	60	4
Bacteroides-Prevotella-Porphyromonas (16S rRNA)	BPP1 BPP2	GGTGTCGGCTTAAGTGCCAT CGGAYGTAAGGGCCGTGC	140	55	1
Lactobacillus spp. (16S rRNA)	LAC-1 LAC-2	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	58	1
Streptococcus spp. (sodA)	Salac5f Salac5r	TCTAGCAAATGCCAATGCTG GATGAGGGCTTGACGAATGT	121	55	6
Enterococcus spp. (16S rRNA)	Ent1 Ent2	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	144	61	1
Bifidobacterium spp. (16S rRNA)	g-BIFID-F g-BIFID-R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	243	58	1
Weissella spp. (16S rRNA)	Weisformod-1 Weisrevmod-1	CACGTGGGWAACCTACCTCTTA ATCTCTTAGTGATAGCAGAACCATC	118	55	6
Leuconostoc spp. (16S rRNA)	Leucformod-1 Leucrevmod-1	GCGGCTGCGGCGTCACCTAG GGNTACCTTGTTACGACTTC	179	55	6
L. acidophilus (16S rRNA)	L-aci I L-aci II	AGCTGAACCAACAGATTCAC ACTACCAGGGTATCTAATCC	200	55	3
L. johnsonii (16S rRNA)	L-joh I L-joh II	GAGCTTGCCTAGATGATTTTA ACTACCAGGGTATCTAATCC	200	55	3
L. mucosae (16S rRNA)	L-muc 2a L-muc 2b	GGCTATCACTTTGGGATGGA ATGGACCGTGTCTCAGTTCC	124	55	6
L. reuteri (16S rRNA)	L-reu 1 L-reu 2	CCCAACTGATTGATGGTGCT GGGCAGGTTACCTACGTGTT	135	55	3
L. amylovorus (16S rRNA)	L-amyl 2a L-amyl 2b	GCGGAACCAACAGATTTACTT GTTTCCAAATGGTATCCCAGACTT	106	58	3

References

1 = Rinttilä et al., 2004; 2 = Malinen et al., 2003; 3 = Walter et al., 2000; 4 = Matsuki et al., 2004. 5 = Song et al., 2004; 6 = unpublished;

Table 4.4:Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 32 days of age (n=6)

	Stomach		**Jejunum**		**Ileum**		**Colon**	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
E. coli/Hafnia/Shigella	6.50 (±0.88)	5.89 (±0.63)	**7.03 (±0.87)^{a}**	**5.80 (±0.82)^{b}**	**8.10 (±0.84)^{a}**	**7.36 (±1.16)^{b}**	**8.68 (±0.75)^{a}**	**8.12 (±0.92)^{b}**
Enterobacteriaceae	**7.20 (±0.65)^{a}**	**7.01 (±0.61)^{b}**	**7.12 (±0.91)^{a}**	**6.19 (±1.22)^{b}**	**9.01 (±0.87)^{a}**	**7.55 (±1.07)^{b}**	**9.35 (±0.70)^{a}**	**8.61 (±0.86)^{b}**
Clostridial cluster I	7.32 (±1.33)	7.55 (±1.20)	8.22 (±1.33)	8.02 (±0.58)	9.32 (±0.52)	9.59 (±0.92)	9.26 (±0.08)	9.64 (±0.12)
Clostridial cluster XIVa	7.46 (±1.06)	8.03 (±1.53)	7.50 (±1.26)	6.76 (±0.22)	7.37 (±0.54)	6.89 (±0.60)	11.88 (±1.00)	11.82 (±0.87)
Clostridial cluster IV	5.76 (±1.00)	5.67 (±0.36)	5.73 (±0.40)	5.80 (±0.34)	6.58 (±0.59)	5.83 (±0.38)	9.91 (±0.25)	9.91 (±0.18)
Bacteroides-Prevotella-Porphyromonas	9.08 (±1.27)	8.89 (±1.36)	8.07 (±1.55)	7.22 (±0.48)	8.60 (±1.75)	7.41 (±1.38)	10.53 (±0.28)	10.54 (±0.25)
Lactobacillus spp.	**10.66 (±0.10)^{a}**	**10.16 (±0.79)^{b}**	**10.34 (±0.19)^{a}**	**9.33 (±0.52)^{b}**	10.22 (±0.30)	9.57 (±1.13)	10.23 (±0.27)	9.88 (±0.18)
Streptococcus spp	8.78 (±0.11)	8.53 (±0.42)	7.81 (±0.25)	7.86 (±0.13)	8.13 (±0.51)	8.20 (±0.42)	9.19 (±0.23)	9.20 (±0.18)
Enterococcus spp.	7.16 (±0.55)	6.64 (±0.75)	6.86 (±0.56)	6.03 (±0.79)	6.93 (±0.71)	6.17 (±0.61)	6.62 (±0.20)	6.42 (±0.39)
Bifidobacterium spp.	8.54 (±)0.68	8.17 (±1.31)	6.89 (±)0.83	6.94 (±0.93)	7.22 (±)1.18	7.33 (±1.15)	7.65 (±1.23)	7.65 (±0.61)
Weissella spp.	5.52 (±0.24)	5.61 (±0.39)	5.45 (±0.21)	5.63 (±0.35)	5.41 (±0.21)	5.67 (±0.25)	5.07 (±0.20)	5.11 (±0.29)
Leuconostoc spp.	5.46 (±0.80)	4.95 (±0.57)	4.58 (±0.46)	4.73 (±0.32)	5.47 (±1.60)	4.84 (±0.80)	5.42 (±0.63)	4.85 (±0.59)
L. acidophilus	7.84 (±0.27)	7.21 (±1.41)	**8.06 (±0.25)^{a}**	**7.04 (±0.78)^{b}**	7.99 (±0.33)	7.48 (±1.29)	7.24 (±0.79)	7.25 (±0.49)
L. johnsonii	6.63 (±1.35)	6.27 (±0.73)	**5.99 (±1.73)^{a}**	**5.52 (±0.70)^{b}**	4.96 (±1.05)	5.39 (±0.85)	6.26 (±1.54)	6.76 (±1.07)
L. mucosae	8.06 (±0.54)	7.69 (±0.82)	**7.44 (±0.36)^{a}**	**5.74 (±0.36)^{b}**	**6.91 (±0.48)^{a}**	**5.85 (±0.50)^{b}**	7.50 (±0.29)	7.22 (±0.17)
L. reuteri	7.94 (±0.30)	7.38 (±0.68)	**7.04 (±0.51)^{a}**	**5.54 (±0.20)^{b}**	**6.62 (±0.20)^{a}**	**5.71 (±0.40)^{b}**	7.17 (±0.45)	6.99 (±0.50)
L. amylovorus	8.86 (±0.44)	8.25 (±1.52)	**9.02 (±0.42)^{a}**	**7.96 (±0.86)^{b}**	9.05 (±0.48)	8.36 (±1.21)	8.47 (±0.75)	8.44 (±0.45)
Sum of detected cell numbers	10.60 (±0.16)	10.08 (±0.61)*	**10.27 (±0.24)^{a}**	**9.36 (±0.33)^{b}**	10.22 (±0.29)	10.35 (±0.89)	11.89 (±0.75)	11.84 (±0.66)

^{a,b} = significantly different between treatments in intestinal segment ($p < 0.05$)

* = trend for difference between treatments in intestinal segment ($p < 0.1$)

Table 4.5: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 39 days of age (n=6)

	Stomach		Jejunum		Ileum		Colon	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
E. coli/Hafnia/Shigella	5.23 (±0.30)	5.71 (±0.79)	6.40 (±0.94)	6.47 (±0.88)	8.28 (±0.59)	8.22 (±0.60)	8.58 (±0.83)	8.43 (±0.42)
Enterobacteriaceae	7.12 (±0.64)	6.83 (±0.81)	7.51 (±0.63)	6.97 (±0.37)	9.41 (±0.59)	8.81 (±0.57)	9.38 (±0.80)	9.04 (±0.61)
Clostridial cluster I	7.61 (±0.37)	7.51 (±0.78)	8.04 (±1.06)	8.05 (±0.60)	9.35 (±0.40)	9.26 (±0.66)	**9.14 (±0.40)^{a}**	**9.52 (±0.34)^{b}**
Clostridial cluster XIVa	7.63 (±1.20)	7.45 (±1.27)	7.15 (±0.74)	6.85 (±0.78)	7.12 (±0.77)	6.96 (±0.55)	11.59 (±0.83)	11.85 (±1.00)
Clostridial cluster IV	5.65 (±0.32)	5.47 (±1.41)	**6.37 (±0.30)^{a}**	**5.54 (±0.68)^{b}**	**7.21 (±0.77)^{a}**	**5.64 (±0.66)^{b}**	9.77 (±0.17)	10.04 (±0.16)
Bacteroides-Prevotella-Porphyromonas	9.26 (±0.48)	9.24 (±0.78)	7.80 (±1.28)	7.20 (±1.17)	8.41 (±0.73)	6.88 (±0.43)	10.32 (±0.14)	10.49 (±0.08)
Lactobacillus spp.	**9.72 (±0.27)^{a}**	**9.28 (±0.50)^{b}**	9.71 (±0.58)	9.53 (±0.43)	9.62 (±0.26)	9.45 (±0.84)	9.95 (±0.59)	9.84 (±0.48)
Streptococcus spp	8.34 (±0.58)	8.28 (±0.20)	8.43 (±0.45)	8.02 (±0.30)	8.62 (±0.72)	8.50 (±0.52)	9.54 (±0.73)	9.29 (±0.73)
Enterococcus spp.	6.54 (±0.30)	6.36 (±0.70)	6.59 (±0.43)	5.83 (±0.42)	6.34 (±0.72)	6.67 (±1.27)	6.75 (±0.42)	6.96 (±0.66)
Bifidobacterium spp.	7.69 (±0.22)	7.95 (±0.96)	6.82 (±0.55)	6.94 (±0.33)	7.36 (±0.42)	7.38 (±0.43)	7.82 (±0.35)	7.94 (±0.30)
Weissella spp.	6.84 (±0.99)	6.91 (±1.26)	6.11 (±0.54)	6.24 (±0.56)	5.96 (±0.37)	6.88 (±0.98)	5.41 (±0.81)	6.83 (±1.05)
Leuconostoc spp.	4.76 (±0.81)	4.85 (±0.77)	4.40 (±0.92)	4.17 (±0.57)	4.92 (±1.20)	5.11 (±1.34)	5.09 (±0.60)	5.20 (±0.74)
L. acidophilus	**7.31 (±0.42)^{a}**	**6.62 (±0.79)^{b}**	7.69 (±0.42)	6.64 (±0.77)	**7.72 (±0.37)^{a}**	**6.55 (±0.83)^{b}**	**7.55 (±0.36)^{a}**	**7.19 (±0.33)^{b}**
L. johnsonii	5.30 (±1.25)	4.87 (±0.62)	5.48 (±1.60)	4.80 (±0.43)	4.53 (±1.32)	4.04 (±0.65)	6.50 (±1.28)	5.81 (±0.73)
L. mucosae	7.25 (±0.36)	7.50 (±0.86)	6.28 (±0.59)	6.00 (±0.46)	6.43 (±0.39)	5.74 (±0.20)	**7.43 (±0.28)^{a}**	**6.88 (±0.66)^{b}**
L. reuteri	6.83 (±0.27)	6.99 (±0.93)	5.87 (±0.54)	5.67 (±0.54)	6.03 (±0.37)	5.44 (±0.25)	6.82 (±0.22)	6.28 (±0.73)
L. amylovorus	**8.22 (±0.41)^{a}**	**7.79 (±0.82)^{b}**	**8.61 (±0.34)^{a}**	**7.65 (±0.74)^{b}**	**8.75 (±0.46)^{a}**	**7.69 (±0.87)^{b}**	**8.85 (±0.42)^{a}**	**8.50 (±0.36)**
Sum of detected cell numbers	9.92 (±0.37)	9.46 (±0.49)*	9.61 (±0.52)	9.29 (±0.25)	9.88 (±0.30)	9.88 (±0.33)	11.44 (±0.69)	11.54 (±0.93)

^{a,b} = significantly different between treatments in intestinal segment ($p < 0.05$)

* = trend for difference between treatments in intestinal segment ($p < 0.1$)

Table 4.6: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 46 days of age (n=6)

	Stomach		Jejunum		Ileum		Colon	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
E. coli/Hafnia/Shigella	5.26 (±0.37)	5.09 (±0.65)	6.17 (±1.19)	5.95 (±0.75)	7.50 (±0.36)	8.01 (±0.39)	7.46 (±0.66)	8.19 (±0.32)
Enterobacteriaceae	6.88 (±0.64)	6.65 (±0.71)	7.29 (±0.36)	7.04 (±0.70)	8.24 (±0.49)	8.78 (±0.65)	8.34 (±0.60)	8.69 (±0.66)
Clostridial cluster I	6.69 (±0.67)	6.73 (±0.81)	7.31 (±0.58)	7.25 (±0.48)	8.72 (±0.70)	9.58 (±0.61)	9.14 (±0.59)	9.26 (±0.46)
Clostridial cluster XIVa	6.75 (±1.23)	6.22 (±0.64)	6.07 (±0.35)	6.08 (±0.92)	6.81 (±0.25)	6.58 (±0.97)	11.73 (±0.84)	11.77 (±0.97)
Clostridial cluster IV	5.58 (±0.65)	4.90 (±0.47)	5.28 (±0.29)	6.00 (±1.22)	6.31 (±0.37)	6.89 (±0.89)	9.61 (±0.15)	9.47 (±0.34)
Bacteroides-Prevotella-Porphyromonas	8.39 (±1.07)	8.25 (±1.05)	7.18 (±0.98)	7.07 (±0.45)	7.46 (±0.85)	7.04 (±0.61)	10.54 (±0.26)	10.42 (±0.11)
Lactobacillus spp.	8.96 (±1.29)	9.02 (±0.37)	**8.86 (±1.40)^{a}**	**8.05 (±0.92)^{b}**	8.93 (±0.88)	8.93 (±0.79)	9.46 (±0.43)	9.52 (±0.53)
Streptococcus spp	8.15 (±0.39)	7.92 (±0.28)	7.74 (±0.16)	7.73 (±0.18)	8.24 (±0.64)	8.36 (±0.41)	8.93 (±0.58)	9.07 (±0.34)
Enterococcus spp.	6.23 (±0.23)	6.06 (±0.85)	5.76 (±0.13)	5.40 (±0.46)	5.67 (±0.50)	6.07 (±0.54)	6.32 (±0.29)	6.43 (±0.43)
Bifidobacterium spp.	6.80 (±1.31)	6.61 (±1.14)	5.66 (±0.98)	5.77 (±0.89)	6.32 (±0.99)	7.11 (±0.71)	6.95 (±0.96)	7.40 (±0.60)
Weissella spp.	6.80 (±1.40)	6.28 (±1.33)	5.65 (±0.61)	5.84 (±0.97)	6.29 (±1.03)	6.71 (±1.25)	6.09 (±1.08)	6.28 (±1.21)
Leuconostoc spp.	4.54 (±0.88)	4.87 (±0.71)	4.79 (±0.72)	4.49 (±0.92)	5.41 (±1.64)	5.52 (±1.53)	5.39 (±1.06)	5.07 (±0.87)
L. acidophilus	6.67 (±1.25)	5.47 (±1.32)	**7.29 (±1.08)^{a}**	**5.70 (±1.29)^{b}**	**7.31 (±0.66)^{a}**	**6.60 (±1.21)^{b}**	**7.19 (±0.35)^{a}**	**6.94 (±0.39)^{b}**
L .johnsonii	5.45 (±1.31)	5.25 (±1.00)	5.07 (±1.38)	4.85 (±0.93)	4.24 (±1.34)	5.00 (±0.62)	6.08 (±1.33)	6.89 (±0.33)
L .mucosae	7.09 (±0.66)	6.10 (±0.99)	**5.79 (±0.68)^{a}**	**4.68 (±1.02)^{b}**	**5.63 (±0.36)^{a}**	**5.45 (±0.49)^{b}**	6.87 (±0.30)	6.87 (±0.47)
L .reuteri	6.75 (±0.59)	5.65 (±1.05)	5.35 (±0.70)	4.68 (±0.75)	5.33 (±0.42)	5.23 (±0.37)	6.35 (±0.50)	6.37 (±0.41)
L .amylovorus	7.58 (±1.22)	6.47 (±1.35)	**8.08 (±0.92)^{a}**	**6.55 (±1.33)^{b}**	**8.18 (±0.61)^{a}**	**7.39 (±1.38)^{b}**	**8.31 (±0.27)^{a}**	**8.05 (±0.39)^{b}**
Sum of detected cell numbers	9.38 (±0.99)	9.37 (±0.55)	9.55 (±0.95)	8.70 (±0.42)*	10.08 (±0.76)	10.29 (±0.52)	11.56 (±0.70)	11.53 (±0.75)

^{a,b} = significantly different between treatments in intestinal segment ($p < 0.05$)

* = trend for difference between treatments in intestinal segment ($p < 0.1$)

Table 4.7: Bacterial cell numbers [log (g wet wt) $^{-1}$] in the intestinal tract of piglets on 53 days of age (n=6)

	Stomach		**Jejunum**		**Ileum**		**Colon**	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
E. coli/Hafnia/Shigella	5.55 (±1.51)	5.20 (±0.75)	5.80 (±0.82)	5.74 (±0.91)	6.76 (±0.57)	7.30 (±0.70)	7.52 (±0.59)	7.11 (±0.84)
Enterobacteriaceae	6.60 (±0.25)	6.99 (±0.54)	6.59 (±0.39)	6.70 (±0.36)	7.34 (±1.50)	7.75 (±0.81)	7.91 (±0.52)	7.82 (±0.78)
Clostridial cluster I	6.67 (±0.96)	6.69 (±0.67)	7.68 (±1.09)	7.79 (±0.67)	9.47 (±0.42)	9.99 (±0.25)	9.28 (±0.29)	9.42 (±0.34)
Clostridial cluster XIVa	6.77 (±0.83)	6.52 (±0.86)	6.54 (±0.56)	6.52 (±0.39)	6.95 (±0.35)	7.52 (±0.78)	11.26 (±0.87)	11.21 (±0.21)
Clostridial cluster IV	5.26 (±0.47)	5.44 (±0.81)	5.68 (±0.51)	5.43 (±0.27)	6.00 (±0.50)	6.44 (±0.76)	9.48 (±0.26)	9.72 (±0.07)
Bacteroides-Prevotella-Porphyromonas	8.54 (±1.15)	8.08 (±0.53)	7.28 (±0.38)	6.86 (±0.82)	6.90 (±0.15)	7.77 (±1.19)	10.44 (±0.19)	10.46 (±0.12)
Lactobacillus spp.	8.68 (±0.80)	8.45 (±0.87)	8.34 (±0.56)	7.41 (±0.17)	**9.05 (±1.21)^{a}**	**7.80 (±0.33)^{b}**	9.35 (±0.37)	9.06 (±0.48)
Streptococcus spp	7.96 (±0.17)	7.99 (±0.26)	7.80 (±0.02)	8.11 (±0.35)	8.00 (±0.30)	8.22 (±0.37)	9.36 (±0.56)	9.11 (±0.58)
Enterococcus spp.	6.36 (±0.69)	6.88 (±1.13)	5.87 (±0.93)	6.47 (±0.99)	6.42 (±0.59)	6.74 (±0.85)	6.78 (±0.66)	7.31 (±0.44)
Bifidobacterium spp.	6.78 (±1.21)	6.41 (±0.82)	5.71 (±0.77)	5.54 (±0.88)	6.27 (±0.80)	6.32 (±0.68)	6.52 (±1.51)	7.42 (±0.38)
Weissella spp.	7.40 (±1.48)	6.45 (±1.32)	6.16 (±0.66)	6.06 (±0.82)	6.22 (±1.09)	6.15 (±1.33)	6.61 (±1.97)	5.87 (±1.06)
Leuconostoc spp.	4.54 (±0.56)	4.99 (±0.68)	4.46 (±0.57)	4.69 (±0.54)	4.71 (±1.41)	5.49 (±0.46)	4.58 (±0.88)	4.93 (±0.81)
L. acidophilus	**5.16 (±1.27)^{a}**	**4.84 (±1.06)^{b}**	**5.41 (±1.22)^{a}**	**3.82 (±0.74)^{b}**	**6.54 (±1.83)^{a}**	**4.55 (±0.51)^{b}**	5.50 (±1.82)	5.26 (±1.12)
L. johnsonii	4.63 (±0.93)	4.72 (±1.02)	4.47 (±0.86)	3.99 (±0.20)	4.71 (±1.29)	4.89 (±1.16)	6.03 (±1.25)	6.35 (±0.79)
L. mucosae	**6.16 (±1.00)^{a}**	**6.07 (±1.16)^{b}**	**4.85 (±0.37)^{a}**	**4.23 (±0.17)^{b}**	**5.69 (±0.89)^{a}**	**4.98 (±0.32)^{b}**	6.78 (±0.49)	6.77 (±0.43)
L. reuteri	5.81 (±0.76)	5.53 (±1.01)	4.72 (±0.66)	4.06 (±0.13)	**5.29 (±0.90)^{a}**	**4.54 (±0.47)^{b}**	6.47 (±0.68)	6.03 (±0.60)
L. amylovorus	**6.55 (±1.21)^{a}**	**6.02 (±1.01)^{b}**	**6.87 (±1.16)^{a}**	**5.24 (±0.49)^{b}**	**7.74 (±1.75)^{a}**	**5.78 (±0.86)^{b}**	7.34 (±1.11)	6.74 (±1.31)
Sum of detected cell numbers	9.32 (±0.73)	8.97 (±0.54)	8.81 (±0.42)	8.74 (±0.65)	9.97 (±0.44)	9.91 (±0.56)	11.25 (±0.65)	11.19 (±0.22)

^{a,b} = significantly different between treatments in intestinal segment ($p < 0.05$)

* = trend for difference between treatments in intestinal segment ($p < 0.1$)

Table 4.8: Bacterial metabolites in the intestinal tract of piglets on 32 days of age [µmol g^{-1} wet weight] (n=6)

	Stomach		Jejunum		Ileum		Colon	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
Acetate	18.20 (±11.19)	12.87 (±10.47)	8.43 (±10.56)	2.33 (±1.79)	20.11 (±16.04)	11.91 (±6.64)	71.93 (±10.71)	68.66 (±6.65)
Propionate	8.99 (±5.94)	6.53 (±5.58)	4.05 (±5.78)	0.46 (±0.60)	**8.23 (±11.01)[a]**	**1.07 (±1.97)[b]**	30.97 (±7.27)	25.77 (±6.25)
i-Butyrate	0.27 (±0.18)	0.20 (±0.20)	0.32 (±0.17)	0.10[1]	**0.24 (±0.08)[a]**	**0.06 (±0.06)[b]**	1.31 (±0.41)	1.07 (±0.37)
n-Butyrate	6.54 (±6.56)	4.17 (±9.52)	1.00 (±2.60)	0.13 (±0.24)	2.59 (±2.92)	0.87 (±1.21)	15.23 (±3.99)	12.86 (±3.03)
i-Valeriate	0.06 (±0.07)	0.03 (±0.04)	0.06 (±0.07)	0.04 (±0.03)	**0.09 (±0.08)[á]**	**0.02 (±0.02)[b]**	1.03 (±0.47)	0.72 (±0.31)
n-Valeriate	1.73 (±1.80)	0.94 (±1.67)	0.36 (±0.84)	0.05 (±0.05)	0.58 (±0.61)	0.17 (±0.07)	**3.99 (±1.46)[a]**	**1.96 (±0.61)[b]**
Total branched chain fatty acids	0.32 (±0.22)	0.17 (±0.22)	0.25 (±0.25)	0.07 (±0.05)	**0.23 (±0.17)[a]**	**0.06 (±0.07)[b]**	2.34 (±0.86)	1.79 (±0.68)
Total volatile fatty acids	35.77 (±23.50)	24.40 (±24.92)	13.95 (±19.54)	2.99 (±2.58)	31.56 (±29.04)	13.93 (±8.70)	124.47 (±20.20)	111.04 (±11.80)
% Acetate	**52.25 (±5.49)[a]**	**66.56 (±18.46)[b]**	72.76 (±12.55)	**80.67 (±10.62)[a]**	**75.74 (±16.82)[b]**	88.26 (±10.25)	**57.96 (±3.37)[a]**	**61.98 (±3.45)[b]**
% Propionate	25.50 (±6.18)	23.66 (±13.05)	21.33 (±11.95)	13.47 (±8.10)	15.96 (±14.83)	6.64 (±9.02)	24.71 (±2.81)	23.10 (±4.29)
% i-Butyrate	0.73 (±0.25)	0.50 (±0.12)	1.07 (±0.32)	2.74[1]	0.43 (±0.01)	0.28 (±0.19)	1.09 (±0.37)	0.96 (±0.33)
% n-Butyrate	16.90 (±8.52)	7.91 (±11.70)	3.40 (±4.04)	3.13 (±2.40)	5.55 (±5.55)	4.58 (±4.45)	12.20 (±1.84)	11.55 (±2.16)
% i-Valeriate	0.21 (±0.22)	0.15 (±0.14)	0.79 (±1.25)	1.62 (±0.91)	1.69 (±3.86)	0.14 (±0.08)	0.87 (±0.42)	0.65 (±0.28)
% n-Valeriate	**4.44 (±2.53)[a]**	**2.01 (±2.05)[b]**	1.85 (±1.50)	1.81 (±1.53)	1.40 (±1.03)	1.23 (±0.57)	**3.18 (±0.79)[a]**	**1.75 (±0.49)[b]**
L- Lactate	25.70 (±11.86)	34.77 (±26.36)	**50.86 (±22.66)[a]**	**21.83 (±13.20)[b]**	48.16 (±22.53)	34.05 (±16.78)	15.22 (±10.07)	19.15 (±7.52)
D- Lactate	29.83 (±18.76)	37.21 (±30.37)	28.80 (±15.91)	15.52 (±12.61)	37.79 (±36.25)	20.77 (±19.65)	13.50 (±8.83)	18.73 (±11.03)
Ammonia	1.70 (±0.77)	1.76 (±0.46)	**4.23 (±2.15)[a]**	**2.70 (±1.44)[b]**	2.45 (±1.44)	2.33 (±1.25)	**11.24 (±2.68)[a]**	**7.00 (±3.24)[b]**
Total metabolites	102.66 (±38.39)	87.80 (±69.89)	**97.11 (±33.26)[a]**	**36.45 (±23.12)[b]**	125.72 (±44.08)	64.29 (±33.76)	158.58 (±31.15)	146.24 (±26.35)

[1] = single value

[a,b] = significantly different between treatments in intestinal segment

Table 4.9: Bacterial metabolites in the intestinal tract of piglets on 39 days of age (n=6)

	Stomach		**Jejunum**		**Ileum**		**Colon**	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
Acetate	14.17 (±11.20)	12.71 (±9.59)	2.93 (±2.73)	2.29 (±2.02)	16.73 (±12.21)	11.34 (±7.46)	68.16 (±13.04)	69.46 (±13.12)
Propionate	5.78 (±5.37)	5.99 (±5.34)	0.94 (±1.51)	0.39 (±0.46)	**4.72 (±5.60)[a]**	**0.23 (±0.20)[b]**	33.87 (±8.26)	28.31 (±5.65)
i-Butyrate	0.20 (±0.10)	0.33 (±0.11)	0.08 (±0.04)	0.09 (±0.02)	0.15 (±0.10)	0.04 (±)	1.07 (±0.30)	0.97 (±0.34)
n-Butyrate	2.51 (±2.84)	4.12 (±4.94)	0.22 (±0.29)	0.11 (±0.09)	0.75 (±0.78)	0.58 (±0.88)	15.94 (±5.61)	12.98 (±3.68)
i-Valeriate	0.05 (±0.04)	0.08 (±0.08)	0.03 (±0.02)	0.15 (±0.22)	0.03 (±0.03)	0.02 (±0.01)	0.67 (±0.34)	0.63 (±0.38)
n-Valeriate	0.68 (±0.63)	1.58 (±1.70)	0.08 (±0.07)	0.06 (±0.02)	0.14 (±0.15)	0.02 (±0.01)	3.70 (±1.55)	2.59 (±1.59)
Total branched chain fatty acids	0.21 (±0.13)	0.36 (±0.16)	0.10 (±0.04)	0.18 (±0.18)	0.11 (±0.13)	0.03 (±0.02)	1.74 (±0.64)	1.61 (±0.72)
Total volatile fatty acids	23.30 (±19.48)	24.47 (±20.62)	4.18 (±4.51)	2.92 (±2.32)	20.22 (±19.08)	12.18 (±8.18)	123.40 (±25.22)	114.94 (±21.09)
% Acetate	64.18 (±6.90)	64.40 (±17.81)	76.59 (±8.33)	76.96 (±16.94)	**79.23 (±11.87)[a]**	**93.77 (±3.45)[b]**	**55.47 (±3.70)[a]**	**60.43 (±3.73)[b]**
% Propionate	23.95 (±4.61)	18.02 (±11.10)	15.97 (±8.02)	11.72 (±7.61)	**26.48 (±28.33)[a]**	**3.05 (±2.84)[b]**	27.50 (±4.14)	24.74 (±3.00)
% i-Butyrate	0.60 (±0.18)	0.87 (±0.14)	3.40 (±4.01)	3.82 (±2.83)	2.02 (±3.20)	0.20 (±)	0.88 (±0.26)	0.84 (±0.23)
% n-Butyrate	8.67 (±5.15)	10.60 (±9.77)	4.11 (±1.16)	3.81 (±1.91)	2.73 (±1.03)	2.89 (±3.71)	12.62 (±2.29)	11.25 (±2.50)
% i-Valeriate	0.23 (±0.19)	1.23 (±2.65)	0.60 (±0.59)	9.47 (±16.57)	0.30 (±0.45)	0.17 (±0.07)	0.55 (±0.30)	0.54 (±0.30)
% n-Valeriate	2.66 (±1.36)	6.30 (±6.15)	2.20 (±1.36)	2.62 (±1.58)	0.72 (±0.63)	0.39 (±0.37)	2.98 (±0.91)	2.21 (±1.16)
L- Lactate	**16.35 (±9.72)[a]**	**14.79 (±14.37)[b]**	30.53 (±10.51)	23.95 (±10.91)	73.74 (±34.43)	35.13 (±25.03)	15.54 (±7.18)	9.34 (±4.98)
D- Lactate	16.82 (±11.43)	16.31 (±15.21)	18.73 (±16.38)	22.43 (±22.55)	33.31 (±21.79)	26.63 (±35.67)	12.18 (±5.38)	7.55 (±6.09)
Ammonia	**3.27 (±0.99)[a]**	**1.72 (±0.74)[b]**	3.82 (±2.09)	2.80 (±2.33)	1.78 (±1.30)	1.97 (±0.70)	6.20 (±2.41)	5.58 (±2.29)
Total metabolites	36.43 (±20.57)	58.05 (±4.53)	47.17 (±30.36)	29.05 (±10.84)	**135.06 (±20.29)[a]**	**39.74 (±19.17)[b]**	**160.48 (±32.51)[a]**	**131.35 (±13.89)[b]**

[a,b] = significantly different between treatments in intestinal segment

Table 4.10: Bacterial metabolites in the intestinal tract of piglets on 46 days of age (n=6) (significant differences highlighted in bold numbers)

	Stomach		**Jejunum**		**Ileum**		**Colon**	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
Acetate	**13.27 (±7.85)[a]**	**5.42 (±3.53)[b]**	**2.09 (±1.66)[a]**	**0.77 (±0.12)[b]**	11.35 (±7.26)	13.56 (±6.30)	72.36 (±5.59)	62.41 (±12.45)
Propionate	4.88 (±2.87)	2.44 (±1.92)	0.33 (±0.32)	0.15 (±0.14)	0.84 (±1.37)	0.12 (±0.07)	**39.23 (±6.45)[a]**	**25.55 (±9.05)[b]**
i-Butyrate	0.16 (±0.06)	0.11 (±0.10)	0.06[1]	n.d.	0.04 (±0.02)	0.03[1]	1.03 (±0.31)	0.97 (±0.19)
n-Butyrate	2.40 (±2.77)	1.05 (±1.08)	0.06 (±0.03)	0.08 (±0.07)	0.53 (±0.81)	0.63 (±0.83)	**18.12 (±5.80)[a]**	**10.72 (±2.62)[b]**
i-Valeriate	**0.03 (±0.01)[a]**	**0.02 (±0.00)[b]**	0.03 (±0.02)	0.01	**0.02 (±0.01)[a]**	**0.04 (±0.03)[b]**	0.60 (±0.38)	0.68 (±0.30)
n-Valeriate	0.89 (±0.78)	0.34 (±0.26)	0.06 (±0.03)	0.06 (±0.03)	0.03 (±0.02)	0.01[1]	**3.31 (±2.29)[a]**	**1.18 (±0.80)[b]**
Total branched chain fatty acids	0.16 (±0.09)	0.08 (±0.09)	0.05 (±0.04)	0.01[1]	0.03 (±0.03)	0.05 (±0.04)	1.63 (±0.68)	1.66 (±0.48)
Total volatile fatty acids	**21.45 (±13.10)[a]**	**9.27 (±6.37)[b]**	2.57 (±1.86)	1.03 (±0.30)	12.76 (±7.85)	14.34 (±7.11)	**134.66 (±16.83)[a]**	**101.52 (±21.94)[b]**
% Acetate	66.47 (±13.23)	62.35 (±12.95)	77.71 (±11.56)	77.69 (±13.72)	88.82 (±10.38)	95.73 (±3.19)	**54.04 (±3.81)[a]**	**61.65 (±1.57)[b]**
% Propionate	21.35 (±13.39)	21.93 (±11.63)	12.67 (±7.03)	13.08 (±9.26)	8.05 (±10.43)	0.96 (±0.55)	**29.07 (±2.44)[a]**	**24.68 (±4.20)[b]**
% i-Butyrate	0.67 (±0.25)	0.73 (±0.68)	5.19[1]		0.23 (±0.18)	0.15[1]	0.77 (±0.25)	1.01 (±0.36)
% n-Butyrate	8.74 (±6.22)	11.96 (±10.87)	3.72 (±2.85)	6.06 (±4.41)	2.83 (±3.42)	3.03 (±3.38)	13.34 (±3.09)	10.79 (±2.83)
% i-Valeriate	0.49 (±0.84)	0.40 (±0.45)	1.81 (±0.98)	1.53[1]	**0.14 (±0.06)[a]**	**0.30 (±0.17)[b]**	0.45 (±0.30)	0.74 (±0.45)
% n-Valeriate	2.97 (±1.84)	3.67 (±3.15)	4.13 (±4.33)	5.88 (±3.07)	0.22 (±0.14)	0.08[1]	2.34 (±1.31)	1.12 (±0.74)
L- Lactate	**31.94 (±26.83)[a]**	**10.08 (±16.26)[b]**	**38.67 (±24.98)[a]**	**21.60 (±13.31)[b]**	**54.61 (±30.66)[a]**	**23.50 (±9.50)[b]**	16.43 (±13.88)	7.12 (±7.36)
D- Lactate	**33.90 (±27.73)[a]**	**14.26 (±22.01)[b]**	18.39 (±19.00)	12.69 (±17.45)	**26.07 (±29.74)[a]**	**11.13 (±10.47)[b]**	13.91 (±13.26)	7.99 (±7.64)
Ammonia	2.10 (±1.80)	1.52 (±1.07)	3.82 (±2.49)	3.43 (±1.41)	2.50 (±0.99)	1.46 (±0.75)	7.95 (±9.12)	4.47 (±3.54)
Total metabolites	**64.33 (±70.44)[a]**	**15.17 (±15.11)[b]**	50.54 (±51.84)	20.74 (±3.20)	**98.40 (±52.62)[a]**	**48.61 (±15.01)[b]**	159.25 (±26.69)	119.11 (±25.94)

[1] = single value; n.d. = not determined; [a,b] = significantly different between treatments in intestinal segment

Table 4.11: Bacterial metabolites in the intestinal tract of piglets on 53 days of age (n=6)

	Stomach		Jejunum		Ileum		Colon	
	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO	Low ZnO	High ZnO
Acetate	**4.05 (±3.21)[a]**	**1.36 (±1.24)[b]**	2.05 (±2.73)	1.99 (±0.98)	12.18 (±6.58)	17.20 (±7.82)	79.48 (±11.61)	68.76 (±8.96)
Propionate	**1.43 (±1.45)[a]**	**0.30 (±0.49)[b]**	0.13 (±0.06)	0.19 (±0.12)	0.16 (±0.08)	0.24 (±0.28)	**36.03 (±8.57)[a]**	**23.86 (±3.71)[b]**
i-Butyrate	0.09 (±0.07)	0.10[1]	n.d.	n.d.	0.08 (±0.04)	0.09 (±0.07)	1.19 (±0.29)	1.15 (±0.23)
n-Butyrate	1.02 (±1.19)	0.11 (±0.14)	0.06 (±0.03)	0.06 (±0.02)	0.48 (±0.50)	0.65 (±0.50)	16.59 (±1.84)	14.33 (±4.22)
i-Valeriate	0.03 (±0.03)	0.02 (±0.02)	0.05[1]	0.09 (±0.09)	0.05 (±0.04)	0.10 (±0.13)	0.73 (±0.29)	0.88 (±0.23)
n-Valeriate	0.33 (±0.44)	0.04 (±0.04)	0.06 (±0.03)	0.04 (±0.02)	0.01[1]	0.03 (±0.01)	**2.91 (±1.35)[a]**	**1.16 (±0.38)[b]**
Total branched chain fatty acids	0.07 (±0.09)	0.04 (±0.06)	0.05[1]	0.09 (±0.09)	0.08 (±0.08)	0.16 (±0.20)	1.92 (±0.55)	2.03 (±0.44)
Total volatile fatty acids	**6.90 (±6.31)[a]**	**1.80 (±1.94)[b]**	2.30 (±2.77)	2.30 (±1.02)	12.89 (±7.09)	18.24 (±8.22)	**136.92 (±19.52)[a]**	**110.13 (±13.54)[b]**
% Acetate	**68.12 (±14.86)[a]**	**83.41 (±9.71)[b]**	82.87 (±7.83)	85.10 (±5.84)	94.76 (±2.54)	94.51 (±4.35)	**58.06 (±2.17)[a]**	**62.55 (±4.54)[b]**
% Propionate	16.59 (±8.09)	10.92 (±7.65)	8.39 (±3.47)	8.67 (±4.87)	1.78 (±1.44)	1.49 (±1.78)	**26.04 (±3.42)[a]**	**21.62 (±1.50)[b]**
% i-Butyrate	0.76 (±0.55)	1.70[1]	n.d.	n.d.	0.53 (±0.04)	0.60 (±0.64)	0.89 (±0.25)	1.05 (±0.25)
% n-Butyrate	**11.18 (±5.94)[a]**	**4.48 (±1.24)[b]**	3.63 (±2.03)	2.94 (±1.77)	2.99 (±2.17)	3.12 (±1.86)	12.33 (±2.23)	12.92 (±2.97)
% i-Valeriate	0.80 (±0.64)	1.11 (±0.47)	3.73[1]	2.87 (±2.66)	0.34 (±0.32)	0.64 (±0.97)	0.56 (±0.30)	0.81 (±0.24)
% n-Valeriate	3.20 (±2.30)	1.46 (±0.48)	4.49 (±2.28)	2.82 (±2.55)	0.04[1]	0.16 (±0.03)	**2.12 (±0.92)[a]**	**1.05 (±0.32)[b]**
L- Lactate	14.61 (±17.46)	9.22 (±9.05)	18.20 (±10.54)	20.32 (±12.19)	66.35 (±51.22)	45.12 (±43.43)	15.12 (±14.16)	8.56 (±6.75)
D- Lactate	17.52 (±19.85)	11.97 (±16.53)	10.98 (±17.07)	20.33 (±19.85)	38.89 (±38.98)	24.77 (±29.13)	11.51 (±14.89)	4.89 (±4.61)
Ammonia	1.34 (±0.56)	1.45 (±0.58)	3.80 (±2.21)	3.84 (±2.65)	2.58 (±1.12)	1.99 (±0.95)	5.44 (±3.50)	4.07 (±3.29)
Total metabolites	15.73 (±18.68)	8.34 (±7.02)	20.42 (±5.81)	16.09 (±5.55)	77.11 (±71.89)	49.43 (±25.13)	149.78 (±22.42)	123.29 (±14.95)

[1] = single value; n.d. = not determined; [a,b] = significantly different between treatments in intestinal segment

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Chapter 5. *Ex vivo* - growth response of porcine small intestinal bacterial communities to pharmacological doses of dietary zinc oxide

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5.1 Abstract

Piglets were fed diets containing 57 (low) or 2425 (high) mg zinc from analytical grade zinc oxide (ZnO) kg^{-1} feed. Digesta samples from the stomach and jejunum of 32, 39, 46 and 53d old animals (n = 6 per group) were incubated in media containing 80, 40, 20 and 0 µg mL^{-1} soluble zinc from ZnO. Turbidity was recorded for 16h and resulting growth curves were used to calculate specific growth rate, maximum growth and lag time. Samples from animals fed the low dietary zinc concentration always showed highest rate of growth and lowest lag times in media without added zinc. However, media supplemented with zinc displayed highest growth rates and lowest lag time in the high dietary zinc group. Maximum growth did not show a clear trend, but specific growth rates and lag time showed significant differences on day 32 and 39 of age. Significant differences for all growth parameters rarely occurred on days 46 and 53 of age. Bacterial growth of digesta samples from the high dietary zinc group was less influenced by zinc and recovered growth more rapidly than in the low dietary zinc group. However, older animals of the low dietary zinc group also showed reduced growth depression. Specific growth rate calculations and bacterial cell numbers from PCR results showed that lactobacilli were most susceptible to zinc, while bifidobacteria and enterococci exhibited increased growth rates in samples from the high dietary zinc treatment. No treatment related differences were observed for clostridial cluster and the *Bacteroides-Prevotella-Porphyromonas* cluster. Enterobacteria from the high dietary zinc treatment showed an increased growth rate at high zinc concentrations in the medium.

This study has shown that the supplementation of feed with a pharmacological dosage of ZnO leads to a reduced *ex vivo* bacterial growth rate of bacteria from the stomach and jejunum of weaned piglets. In view of the rapid bacterial adaptation to dietary zinc, the administration of ZnO in feeds for weaned piglets might only be beneficial in a short period after weaning.

5.2 Introduction

The beneficial effects of pharmacological doses of zinc oxide (ZnO) in animal nutrition, especially piglet nutrition, are well documented [1], [2]. ZnO leads to improved performance and animal health, particularly in the critical period after weaning. The often observed reduction of post-weaning diarrhea in piglets fed zinc supplemented diets led to the belief that ZnO acts bactericidal. However, relative to the wide-spread use of high dietary zinc levels in animal nutrition, there are surprisingly few *in vivo* studies available on the influence of zinc on intestinal bacteria. In a study by Hojberg et al. [3] especially lactobacilli colony counts were reduced and increased coliform colony counts were observed. Results on bacterial cell numbers from the same animals used in this study also show that Lactobacilli were reduced, but enterobacteria were only reduced in the first week after weaning [4]. Finally, sequencing studies on the effect of high dietary zinc on the microbiota in piglets have shown that the enterobacterial diversity increases due to high dietary zinc [5].

ZnO has a low water solubility (16mg·L^{-1}; solubility product constant: 3.86 x 10^{-10}), but as an amphoteric molecule ZnO displays higher solubility at acidic and alkaline conditions. Due to low stomach pH, ZnO solubility is increased after feed intake and rather high percentages of Zn^{2+} ions can be observed in the stomach of piglets (54% at 164 ppm ZnO kg^{-1} diet) [6]. As a consequence, free Zn^{2+} ions may reach the small intestine and act bactericidal.

The stomach and small intestine of piglets harbor a large variety of bacterial species, dominated by lactic acid bacteria, especially lactobacilli [5], [7], [8], [9]. A recent study on the antibacterial activity of ZnO *in vitro* has shown that among the lactic acid bacteria, all tested *Leuconostoc* spp. and *Weissella* spp. were highly resistant, but some *Lactobacillus* spp., *Streptococcus* spp. and *Enterococcus* spp. strains showed lower resistance [10]. Therefore, a shift in the composition of lactic acid bacteria can be expected *in vivo.* In fact, this has been shown for the ileum of 56d old piglets in a pyrosequencing study [11].

Previous studies on the influence of zinc oxide in weaned piglets focused on the porcine microbiota at a single time point. No studies are available that tested the sensitivity of bacterial populations against a range of zinc concentrations and their potential to adapt to the presence of dietary zinc at pharmacological dosage. Therefore, this study was conducted to elucidate the effect of zinc oxide on the development of the bacterial growth response and

possible adaptation to dietary zinc in the stomach and small intestine of weaned piglets under *ex vivo* conditions.

5.3 Material and methods

The study was approved by the local state office of Health and Social Affairs 'Landesamt für Gesundheit und Soziales, Berlin' (LaGeSo Reg. Nr. 0347/09).

Animals and Housing

Purebred landrace piglets were weaned at 26 ± 1 days of age with a mean body weight of 7.2 ± 1.2 kg and randomly allocated into the treatment groups balancing for gender, litter and body weight. Treatment groups were assigned as low dietary zinc (57 $mg \cdot kg^{-1}$ feed) and high dietary zinc (2425 $mg \cdot kg^{-1}$ feed). Animals were housed in pens (n = 2 per pen) with straw bedding and *ad libitum* access to feed and water. The room temperature was 26 °C at stabling, and was incrementally decreased to 22 °C within the first week after weaning according to the standard conditions in the institute. The humidity was kept constant and the light program ensured a 16 hour light and an 8 hour dark phase. No antibiotics were administered before and during the experiment.

Diets

Piglets received a mash starter diet until 53^{th} d of life (Supplemental Table 1). The zinc level of the diet was adjusted with ZnO to approximately 50 or 2500 mg zinc per kg feed with analytical grade zinc oxide (Sigma-Aldrich, Taufkirchen, Germany). The dietary zinc levels were confirmed by analysis via atomic absorption spectrometry [12] and yielded 57 and 2425 mg total zinc per kg dry matter.

Bacteriological Media

A nutrient rich medium (reinforced clostridial medium, LAB022) for cultivation of anaerobic bacteria was used in this study. This medium has been shown to allow growth of a broad range of bacterial strains [9]. Zinc oxide supplemented media were generated by saturating the medium with 10g analytical grade ZnO (Sigma-Aldrich, Taufkirchen, Germany) per

100mL medium. After stirring for 30 min at room temperature, the medium was autoclaved (20 min, 121 °C, 2 bar) and centrifuged (15 min, 18.500xg, 4 °C). Supernatants were collected and total Zn content was determined by atomic absorption spectroscopy [13]. Non supplemented medium was then used to dilute the zinc concentration to 80, 40 and 20 $\mu g \cdot mL^{-1}$. The zinc supplemented media were prepared fresh one day in advance to each sampling day. On sampling days, microtiter plate wells (U-form) were filled (200 µl) with the respective freshly prepared media (0, 20, 40, 80 $\mu g \cdot mL^{-1}$) in a glove box under anaerobic conditions (95% N_2/ 5% H_2).

Sampling and Incubation

Digesta samples were taken from animals (n = 6 per group and day) at the age of 32 ± 1, 39 ± 1, 46 ± 1 and 53 ± 1 days of life. After slaughter, individual stomach and mid-jejuna contents were filled into 2mL plastic tubes and immediately transferred into anaerobic glove boxes. The digesta samples were diluted in zinc free medium (1:5 vol/vol), mixed gently and left to sediment for 5 min. Supernatants were drawn with cut tips and again diluted in non-zinc supplemented medium (1:10 vol/vol). These sample dilutions were then inoculated in triplicate in prepared microtiter plates, sealed with air tight membranes (Viewseal, Greiner) and transferred into a microtiter plate reader with anaerobic incubation capacity (Tecan Infinite200Pro, Crailsheim, Germany). Non inoculated wells with media served as controls. Plates were incubated for 16 h at 37 °C and turbidity was measured at 690 nm every 5 min.

Calculation of Growth Parameters

Turbidity data and bacterial cell numbers were transformed into growth curves and subjected to a nonlinear regression using the logistic sigmoid 3 parameter curve fit equation. Individual growth curves were inspected for goodness of fit and curve fits below $r^2 = 0.98$ were rejected from further analysis. Lag times, specific growth rate and maximum growth were calculated for each individual growth curve of the turbidity data. Specific growth rates were determined for bacterial cell numbers. To study the growth differences between zinc free and zinc supplemented media of the same digesta sample, the turbidity measurements of incubations in zinc free medium was subtracted from zinc supplemented medium at each time point of the incubation and given as subtractive growth values.

Determination of Bacterial Cell Numbers from incubated Samples

DNA extraction: After incubation, selected samples of 0 and 80 µg·mL^{-1} zinc media (n=4 per treatment) were quantitatively removed from microtiter wells and centrifuged (19.000 *g*, 10 min, 37 °C). Resulting supernatants were replaced with 200 µl RNAlater (Qiagen, Hilden, Germany) and the suspension was frozen at -30 °C until further analysis. After thawing, a subsample of 100 µl suspension was used to extract DNA with a commercial DNA extraction kit (Macherey-Nagel, Dueren, Germany).

Realtime PCR – Assays: Primer sequences and annealing temperatures are given in Supplemental Table 2. All primers were purchased from MWG Biotech (Straubing, Germany). A Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) was used for PCR amplification and fluorescent data collection. The master mix consisted of 12.5 µL Brilliant II SYBR® Green QPCR Master Mix with Low ROX (Stratagene, Amsterdam, Netherlands), 0.5 µL of each primer (10 µM) and 10.5 µl water. One µl sample was added before PCR amplification. All amplification programs included an initial denaturation step at 95 °C for 10 min to activate the polymerase. All PCR programs featured an annealing time of 60 sec and a 60 sec extension at 72 °C.

Quantification of Fluorescent Signals

A detailed description of the quantification procedure is given by Vahjen et al. [14]. In short, overnight cultures from a wide range of bacterial species and known cell numbers (10^9 cells·mL^{-1}) were combined according to their respective phylogenetic groups. After extraction and purification with the same DNA extraction protocol, these extracts were used as PCR calibration samples and results were expressed as cell number per mL sample.

Denaturing gradient gel electrophoresis

DNA extracts of the replicates from each sample were pooled and subjected to a qualitative determination of bacterial diversity using the DGGE. PCR was performed with a commercial Multiplex PCR kit (Qiagen, Hilden, Germany). Each PCR mixture contained 0.5 µM of a eubacterial primer pair [15] or a primer pair for enterobacteria developed at our institute, respectively (Table 5.5); 100 ng of purified DNA and equal amounts of sterile distilled water and master mix. Amplification of the ribosomal polymerase subunit beta region was carried

out in a T1 Thermocycler (Biometra, Göttingen, Germany) with 30 cycles of the following program: initial activation step at 95 °C for 10 min followed by a denaturation step at 95 °C for 15 sec, an annealing step at 50 °C for 1 min, an elongation step at 72 °C for 1 min and a final elongation at 72 °C for 10 min.

The Ingeny phorU DGGE system (Ingeny, Goes, Netherlands) was used for subsequent nucleotide sequence-specific separation of PCR amplicons using a 30–55% urea gradient in 6% polyacrylamide gels. Electrophoresis was performed at 60 °C for 20h at 100 V. Gels were scanned after silver staining and analyzed by the Phoretix 1D Advanced version V11.2 software package (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Statistical Analysis

Statistical analysis was carried out with SPSS 19.0 (SPSS Inc., Illinois, USA). Arithmetic means and standard deviations were calculated for lag times, specific growth rate, maximum growth as well as specific growth rate and initial and final cell numbers for bacterial cell numbers. An unpaired two sample t-test was used to determine statistical significance between zinc concentration regarding lag times, specific growth rate, maximum growth for trial groups, age and intestinal segment as well as for comparison of growth response data after subtraction of turbidity in non-zinc supplemented media. Specific growth rates from bacterial cell numbers were analyzed by the non-parametric Mann-Whitney-U test. In all cases, a p-value ≤ 0.05 was considered as significantly different.

5.4 Results

All animals remained clinically healthy during the entire period. Diarrhea occurred only occasionally with no differences between treatments. The average daily weight gain (ADG) and average daily feed intake (ADFI) were higher ($P<0.05$) in the high dietary zinc group during the first week as compared to the other group, but this effect almost reversed after 3 weeks with higher ADG in the low ZnO group [14].

All incubations in zinc free medium showed typical sigmoid growth curves with varying lag times, exponential growth and stationary phase. Figure 5.1 displays an example of the *ex vivo* bacterial growth in stomach digesta samples of 32d old piglets fed low or high dietary zinc

when incubated without or with 80 µg total zinc·mL^{-1} medium. In zinc free medium, digesta samples from animals fed the low dietary zinc concentration (57 ppm) showed a shorter lag time, but no higher specific growth rate (i.e. slope during exponential growth) than digesta samples from animals fed the high dietary zinc concentration (2425 ppm). Maximum growth after 16h incubation was identical for both trial groups. At 80 µg·mL^{-1} total zinc in the medium, the lag time of the low dietary zinc group was still shorter, but specific growth rate as well as maximum growth were higher for the high dietary zinc group. This course of growth was also similar in jejuna digesta samples from 32d old animals, i.e. longer lag times and higher maximum growth as well as higher specific growth rates were observed in the high dietary zinc group. However, significant differences were less frequent than in stomach digesta samples (Table 5.1). Table 5.1 also shows that significant differences were more frequent in younger animals, although numerical differences between trial groups still occurred for the 46th and 53rd day of life.

In order to evaluate the influence of zinc on the bacterial growth potential in a time dependent manner, turbidity from non-zinc supplemented medium was subtracted from respective data of incubations with zinc supplemented media from day 32 to day 53 to yield subtractive growth values. Table 5.2 shows that bacteria from the high dietary zinc group were less influenced at the highest zinc concentration in the medium and recovered growth more rapidly than bacteria from the low dietary zinc group. For instance, the highest zinc concentration showed significant growth differences on the 32nd day of life from 4 to 10h incubation in stomach digesta samples and from 6 to 10h in jejuna digesta samples. Compared to the highest concentration, the influence of zinc decreased at a zinc concentration of 40 µg·mL^{-1} (Table 5.6). At 20 µg zinc·mL^{-1} medium only a slight numeric growth depression was visible in stomach digesta samples (Table 5.6).

Stomach digesta samples from older animals were less influenced by zinc in the high dietary zinc group. Also, growth depression in the low dietary zinc group on the 53rd day of life was less pronounced than on the 46th day of life. A numeric reduction of the growth depression was especially visible for jejuna digesta samples at lower zinc concentrations. However, the low dietary zinc group was also less influenced in older animals.

As the most pronounced differences in bacterial growth behavior were observed during the first three weeks of the feeding trial, incubated samples from those animals were analyzed for

the main bacterial groups present in the stomach and jejunum of piglets. The specific growth rate of selected bacterial groups as determined from qPCR cell numbers are shown in Table 5.2. Highest growth rates were generally observed for enterobacteria, followed by bifidobacteria and lactobacilli. Bacteria belonging to the *Bacteroides-Prevotella-Porphyromonas* cluster did not grow well on all sampling days and intestinal segments, as evidenced by very low to negative growth rates. Similarly, the clostridial cluster IV showed low growth rates on 32 and 39 days of life.

Bifidobacteria and enterococci showed significantly or numerically increased growth rates in samples from piglets of the high dietary zinc treatment on all sampling days. On the contrary, lactobacilli always showed reduced growth rates in the high dietary zinc treatment. Enterobacteria displayed lower growth rates in samples from the high dietary zinc treatment in non-zinc supplemented media, but the same samples exhibited higher growth rates in media supplemented with zinc. The comparison of zinc concentrations in the medium and sample origin also showed that the clostridial cluster I and XIVa always showed higher growth rates in media supplemented with zinc.

Initial and final cell numbers of selected bacterial groups are shown in Table 5.8 and Table 5.9. The dominating bacterial groups in non-zinc supplemented media after 16h incubation were lactobacilli, bifidobacteria and enterobacteria. However, lactobacilli already dominated the initial total cell numbers. Compared to non-zinc supplemented media zinc supplementation led to decreased lactobacilli counts after 16h incubation, but bifidobacteria and enterobacteria generally showed a much more pronounced gain in cell number. The increase in cell numbers for other bacterial groups were much less pronounced, especially the *Bacteroides-Prevotella-Porphyromonas* cluster and the clostridial cluster IV did not increase in cell number in samples from day 32 and day 39.

The qualitative analysis of the eubacterial composition after 16 h incubation did not show differences between media supplemented with 0 or 80 mg mL^{-1} zinc (data not shown).

However, the similarity of enterobacteria showed a clear clustering for DGGE profiles of sample extracts from the low and high dietary zinc treatment, respectively (Figure 5.2). Furthermore, all DGGE profiles form the high dietary zinc treatment formed distinct subclusters according to sampling day. This was less evident for profiles from the low dietary zinc treatment. Table 5.9 shows that the diversity indices for the enterobacterial DGGE

profiles were modified due to the presence of zinc in the growth medium. The number of enterobacterial species (i.e. richness) was always higher in zinc supplemented medium, regardless of dietary treatment. In turn, the Shannon index showed a higher diversity (except on day 32 in the jejunum of the low dietary zinc treatment).

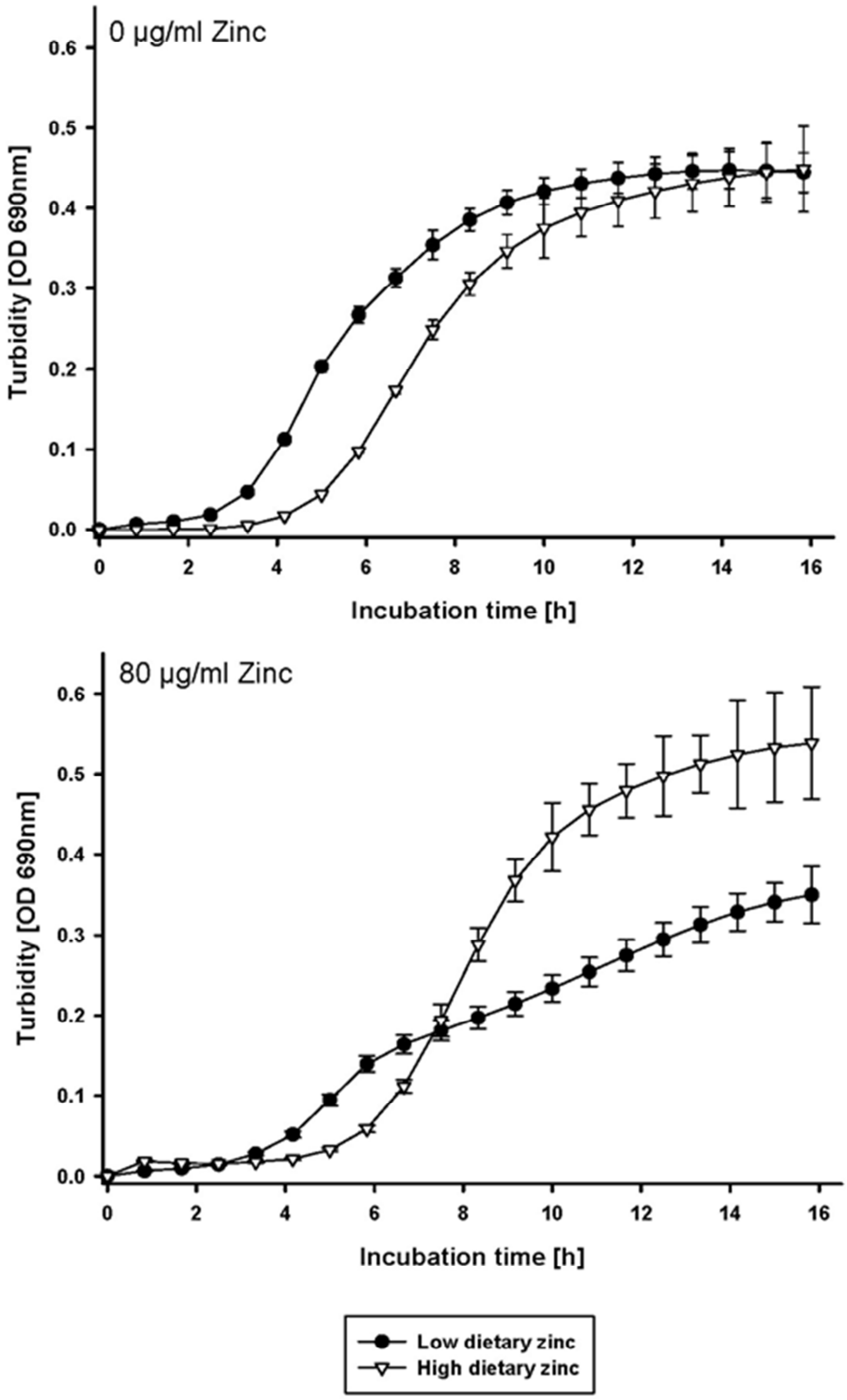

Figure 5.1: Example for the *ex vivo* growth of bacteria in stomach digesta samples of 32d old piglets fed low or high dietary zinc in non-zinc supplemented medium (black circle) or medium supplemented with 80 µg zinc mL^{-1} (white triangle)

Table 5.1: Influence of dietary zinc on the growth parameters of bacteria in the stomach and jejunum digesta samples of piglets after *ex vivo* incubation in zinc supplemented media

			Slope			Max. OD [690nm]			Lag [h]		
Age	Segment	Zinc [µg/ml]	Low zinc	High zinc	p-value	Low zinc	High zinc	p-value	Low zinc	High zinc	p-value
35	Stomach	0	0.461	0.462	0.963	0.52	0.59	0.109	**3.48**[a]	**4.70**[b]	0.012
		20	**0.387**[a]	**0.491**[b]	0.018	**0.54**[a]	**0.77**[b]	0.026	**3.65**[a]	**5.02**[b]	0.001
		40	**0.355**[a]	**0.548**[b]	0.004	**0.43**[a]	**0.68**[b]	0.000	**4.39**[a]	**5.21**[b]	0.037
		80	**0.310**[a]	**0.652**[b]	0.011	**0.41**[a]	**0.64**[b]	0.007	**5.25**[a]	**5.56**[b]	0.076
	Jejunum	0	0.701	0.746	0.46	0.54	0.61	0.001	4.51	5.17	0.176
		20	**0.608**[a]	**0.769**[b]	0.084	**0.57**[a]	**0.70**[b]	0.004	4.35	4.99	0.414
		40	0.633	0.794	0.351	0.51	0.69	0.129	4.86	5.35	0.552
		80	**0.611**[a]	**0.792**[b]	0.059	**0.46**[a]	**0.67**[b]	0.006	5.17	5.83	0.555
42	Stomach	0	**0.524**[a]	**0.424**[b]	0.018	0.61	0.59	0.008	3.08	4.91	0.006
		20	0.447	0.492	0.156	**0.58**[a]	**0.70**[b]	0.004	**3.07**[a]	**3.75**[b]	0.004
		40	0.413	0.498	0.105	**0.50**[a]	**0.72**[b]	0.005	**3.62**[a]	**4.26**[b]	0.033
		80	**0.383**[a]	**0.494**[b]	0.005	**0.49**[a]	**0.68**[b]	0.023	**4.72**[a]	**5.75**[b]	0.073
	Jejunum	0	0.560	0.616	0.141	0.55	0.47	0.195	**3.23**[a]	**3.77**[b]	0.019
		20	0.515	0.557	0.297	**0.46**[a]	**0.62**[b]	0.081	**3.42**[a]	**4.00**[b]	0.062
		40	0.489	0.562	0.113	0.51	0.66	0.614	3.87	4.24	0.249
		80	0.547	0.649	0.941	0.43	0.61	0.219	4.92	5.21	0.469
49	Stomach	0	0.530	0.480	0.250	0.55	0.52	0.400	4.25	4.94	0.287
		20	0.514	0.482	0.423	0.52	0.58	0.154	4.55	4.29	0.607
		40	0.514	0.500	0.727	0.49	0.54	0.350	4.98	4.53	0.427
		80	0.506	0.556	0.718	0.43	0.48	0.891	5.09	4.94	0.770
	Jejunum	0	0.521	0.508	0.691	0.47	0.45	0.348	4.38	4.40	0.969
		20	0.519	0.482	0.423	0.52	0.54	0.154	4.55	4.29	0.606
		40	0.514	0.500	0.727	0.43	0.48	0.995	4.98	4.53	0.427
		80	0.526	0.536	0.718	0.40	0.52	0.891	5.09	4.94	0.770
56	Stomach	0	0.503	0.453	0.114	0.50	0.58	0.903	**4.79**[a]	**5.09**[b]	0.007
		20	0.487	0.553	0.197	0.52	0.55	0.723	4.68	5.14	0.373
		40	0.456	0.568	0.171	0.50	0.57	0.136	4.83	5.20	0.056
		80	**0.437**[a]	**0.597**[b]	0.031	0.45	0.54	0.909	5.24	5.33	0.851
	Jejunum	0	0.590	0.543	0.140	0.68	0.63	0.196	**4.88**[a]	**5.01**[b]	0.015
		20	0.588	0.643	0.448	0.54	0.57	0.818	4.06	5.07	0.211
		40	0.606	0.663	0.438	0.52	0.58	0.342	**4.47**[a]	**4.21**[b]	0.012
		80	**0.619**[a]	**0.752**[b]	0.001	0.41	0.55	0.64	4.43	4.67	0.124

[a, b] Means within rows for each parameter with different superscripts differ ($P < 0.05$).
Significant differences ($P < 0.05$) between treatments are highlighted in bold

Table 5.2: Bacterial growth response to zinc [80 µg mL^{-1}] supplemented medium in stomach and jejunum digesta samples of piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media)

		Stomach			Jejunum		
Day	Time [h]	low zinc	high zinc	p-value	low zinc	high zinc	p-value
32	2	-0.015	-0.008	0.139	0.001	-0.001	0.756
	4	**-0.113^{a}**	**-0.047^{b}**	0.012	-0.051	-0.016	0.084
	6	**-0.179^{a}**	**-0.060^{b}**	0.014	**-0.130^{a}**	**-0.089^{b}**	0.022
	8	**-0.105^{a}**	**-0.011^{b}**	0.008	**-0.161^{a}**	**-0.038^{b}**	0.026
	10	**-0.013^{a}**	**0.009^{b}**	0.023	**-0.121^{a}**	**-0.057^{b}**	0.008
	12	0.009	0.015	0.264	-0.058	-0.050	0.164
	14	0.046	0.064	0.235	-0.018	-0.021	0.582
	16	0.047	0.080	0.101	0.001	-0.004	0.704
39	2	0.003	-0.002	0.259	0.001	-0.001	0.442
	4	**-0.078^{a}**	**-0.043^{b}**	0.041	-0.015	-0.012	0.284
	6	**-0.164^{a}**	**-0.094^{b}**	0.005	**-0.113^{a}**	**-0.034^{b}**	0.044
	8	**-0.166^{a}**	**-0.105^{b}**	0.033	**-0.179^{a}**	**-0.104^{b}**	0.007
	10	**-0.149^{a}**	**-0.095^{b}**	0.042	**-0.105^{a}**	**-0.043^{b}**	0.012
	12	**-0.118^{a}**	**-0.065^{b}**	0.009	-0.023	-0.031	0.422
	14	-0.069	-0.053	0.048	0.033	0.040	0.523
	16	-0.060	-0.032	0.411	0.064	0.076	0.446
46	2	0.003	-0.004	0.452	0.007	0.008	0.862
	4	-0.116	-0.088	0.162	-0.004	-0.001	0.795
	6	-0.050	-0.035	0.206	-0.060	-0.053	0.387
	8	**-0.123^{a}**	**-0.073^{b}**	0.042	**-0.047^{a}**	**-0.010^{b}**	0.039
	10	**-0.105^{a}**	**-0.062^{b}**	0.031	-0.024	-0.009	0.082
	12	**-0.071^{a}**	**-0.030^{b}**	0.022	0.039	0.011	0.288
	14	-0.049	-0.019	0.128	0.050	0.032	0.323
	16	-0.030	-0.015	0.524	0.064	0.043	0.594
53	2	0.000	0.007	0.397	0.008	0.005	0.362
	4	-0.002	-0.004	0.229	-0.007	-0.004	0.114
	6	-0.033	-0.027	0.172	-0.016	-0.019	0.470
	8	-0.041	-0.035	0.382	-0.047	-0.024	0.089
	10	-0.034	-0.025	0.227	-0.024	-0.003	0.084
	12	-0.018	-0.017	0.693	0.008	0.007	0.824
	14	-0.015	-0.011	0.552	0.023	0.042	0.611
	16	-0.020	0.011	0.419	0.045	0.064	0.403

[a, b] Means within rows and intestinal segment with different superscripts differ ($P < 0.05$, t-test). Significant differences between treatments are highlighted in bold.

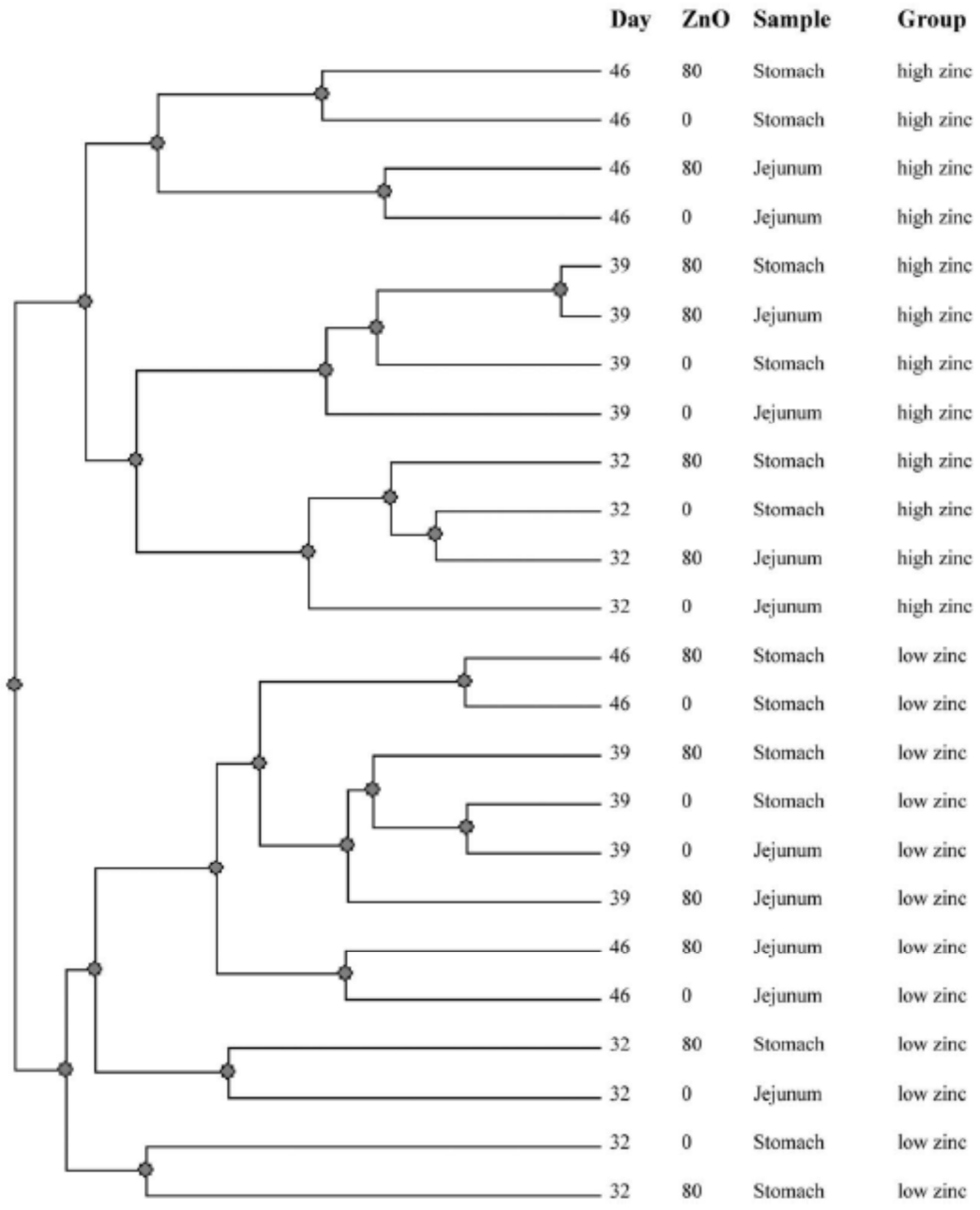

Figure 5.2: Similarity analysis of DGGE profiles from stomach and jejunum samples of piglets fed low or high dietary zinc after 16 h incubation in control- (0) or zinc supplemented medium (80) (UPGMA method).

5.5 Discussion

This study used an *ex vivo*-incubation model of gastric and jejuna digesta in complex medium to examine the influence of dietary zinc oxide on the growth potential of porcine stomach and small intestinal bacteria. The use of ZnO supplemented media by centrifugation of saturated media ensured that only soluble Zinc was present during the incubations with intestinal digesta samples. Since ZnO solubility increases at acidic pH, the use of ZnO suspensions would have led to increased zinc content at later stages of the incubation due to the formation of volatile fatty acids and lactate. It is however likely that the centrifugation step also removed some proteins as well as phosphates, because it is known that ZnO complexes with peptides or phosphates [15]. Nevertheless, as growth occurred even at the highest zinc concentration, nutrient composition must have been sufficient to support bacterial growth for some bacterial groups. Data from qPCR shows that the Bacteroides-Prevotella-Porphyromonas cluster as well as the Clostridium cluster IV did not grow well under the *ex vivo* conditions. This may be due to bacterial competition during the incubation, as enterobacteria also showed low initial cell numbers, but final cell numbers were generally 4 to 5 log units higher. The lower initial bacterial growth (longer lag time) in digesta from animals fed a high dietary zinc oxide concentration confirms the antibacterial effect of dietary zinc oxide in piglets.

However, the often observed compensation of growth at the end of the incubation (higher maximum OD) in zinc supplemented media may be due to enhanced growth of a few zinc resistant species. In this experiment, this could be seen for enterobacteria and bifidobacteria, which showed lower initial cell counts than the dominating lactobacilli, but due to a higher growth rate gained relatively more cell mass at the end of the incubation.

The trend for higher maximum OD was only observed in younger animals of the high dietary zinc group and although numerical differences still continued in 46 and 53 day old animals, significant differences were only found for younger animals. Furthermore, specific growth rates (slope of exponential growth) also followed the same trend throughout the feeding trial, as significantly higher specific growth was often observed during the first two weeks of the trial in digesta samples of the high dietary group. Therefore, it is probable that due to reduced competition zinc resistant species rapidly outgrew zinc sensitive species after an initial growth depression. It remains unknown which types of bacteria gained growth

advantage or were suppressed by zinc in this artificial system. Unfortunately, the high sample number prohibited detailed analysis by plating of molecular methods. However, it is likely that the digesta samples contained large numbers of lactic acid bacteria and enterobacteria, as the stomach and proximal small intestine of piglets is typically dominated by these bacterial groups [5], [7], [8], [9]. Although the employed medium supports the growth of a wide variety of bacteria, sampling and pre-incubation processing may have excluded growth of strict anaerobic species. Furthermore, it is assumed that much higher cell numbers of lactic acid bacteria will outgrow strict anaerobes in an *in vitro* system.

In a recent *in vitro* study, Liedtke and Vahjen [10] tested a broad range of intestinal bacterial strains for their minimal inhibitory concentrations against zinc oxide. That study did not find a clear phylogenetic pattern regarding zinc oxide inhibition, as all bacterial groups showed some members with higher or lower resistance against zinc oxide. The authors concluded that the antibacterial activity of zinc was species specific. Most enterobacteria strains and lactic acid bacteria were not particularly sensitive in that study. Interestingly, *Lactobacillus amylovorus*, one of the dominating *Lactobacillus* spp. in the small intestine of piglets [5], [8] showed the lowest zinc resistance among the tested lactobacilli. This corresponds to results of the *in vivo* study by Hojberg et al. [3] as well as to data for bacterial cell numbers of the same animals [4]. Consequently, it could be speculated for this *ex vivo* study that a reduction of a dominant *Lactobacillus* species occurred, which would leave an increased amount of substrates for zinc resistant lactic acid bacteria or other bacteria. In addition, as *in vivo* studies also showed an increased diversity of enterobacteria [5], [14] it is likely that enterobacteria gained growth advantage in the employed *ex vivo* system.

Heavy metal resistance mechanisms in general seem to be more efficient in Gram-negative bacteria. Gram-negative bacteria rely on specific proton-cation antiporter efflux systems for heavy metals, while Gram-positive bacteria only use P-type efflux ATPases to expel zinc from their cells [18]. Genes for proteins of the Resistance-nodulation-cell-division transporter protein family, specifically for the heavy-metal efflux protein family are much more abundant in Gram-negative bacteria than in Gram-positive bacteria [16]. Thus, enterobacteria may possess more efficient systems to expel intracellular zinc.

The higher initial growth depression also indicates that bacteria of the high dietary zinc group were in a more stressed physiological state than bacteria in digesta samples of the low

dietary zinc group, because the lag time in a growth curve relates directly to the physiological fitness of a bacterial population. As this effect diminished in older animals, the degree of resistance to zinc must have increased in bacteria from animals of the high dietary zinc group, i.e. an adaptation of those bacterial populations occurred during the feeding trial. This was also shown for enterobacteria and Lactobacillus spp. in a study, in which the same animals were used to determine bacterial cell numbers [4]. Similar observations have also been reported for microbiota in soil [20].

However, the antibacterial effects of zinc also diminished over time in digesta samples of the low dietary zinc group. Therefore, it can be assumed that due to the development of a more diverse microbiota, an adaptation also occurred in the low dietary zinc group, though at a later time.

5.6 Conclusions

The results of this study have shown that zinc from zinc oxide leads to bacterial growth depression in the stomach and jejunum of weaned piglets in the early phase after weaning. Bacterial adaptation to zinc occurs within 2 to 3 weeks in animals given a diet with a pharmacological zinc oxide dosage. However, bacterial populations in older animals fed a diet with low dietary zinc oxide also seem to adapt to the presence of zinc during *ex vivo* growth. Based on these observations, the administration of ZnO in feeds for weaned piglets seems to be effective only within short periods. Therefore, the use of ZnO could be restricted to the critical time directly after weaning without compromising the beneficial effect on animal health.

5.7 Acknowledgements

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Supporting Information

Table 5.3: Composition of diets (as-is basis)

Item	
Ingredients, g/kg	
Wheat	380
Barley	300
Soybean meal	232
Corn starch/ zinc oxide[1]	10
Limestone	20
Monocalcium phosphate	20
Mineral & Vitamin Premix[2]	15
Soy oil	17.5
Salt	2.0
Lysine HCl	2.5
Methionine	1.0
Calculated contents	
Dry matter, g/ kg	879
ME, MJ/kg	13.0
Crude ash, g/ kg	81
Crude protein, g/ kg	194
Crude fiber, g/ kg	36
Ether extract, g/ kg	34
Starch, g/ kg	376
Lysine, g/ kg	11.7
Methionine, g/ kg	4.0
Threonine, g/ kg	7.2
Tryptophane, g/ kg	2.4
Calcium, g/ kg	11.0
Phosphorus, g/ kg	8.0
Sodium, g/ kg	3.1
Magnesium, g/ kg	2.2
Zinc, mg/kg[3]	34
Iron, mg/kg	309
Manganese, mg/kg	81
Copper, mg/kg	18

[1] Corn starch in the basal diet was partially replaced in the diets containing 50 and 2500 mg/kg zinc with analytical grade zinc oxide (Sigma Aldrich, Taufkirchen, Germany) to adjust for the zinc level.
[2] Mineral and Vitamin Premix (Spezialfutter Neuruppin Ltd., Neuruppin, Germany), providing per kg feed: 1.95 g Na (as sodium chloride), 0.83 g Mg (as magnesium oxide), 10,500 IU Vitamin A, 1,800 IU Vitamin D3, 120 mg Vitamin E, 4.5 mg Vitamin K3, 3.75 mg Thiamine, 3.75 mg Riboflavine, 6.0 mg Pyridoxine, 30 µg Cobalamine, 37.5 Nicotinic acid, 1.5 mg Folic acid, 375 µg Biotin, 15 mg Pantothenic acid, 1200 mg Choline chloride, 75 mg Fe (as Iron-(II)-carbonate), 15 mg Cu (as Copper-(II)- sulfate), 90 mg Mn (as Manganese-(II)-oxide), 675 µg J (as Calcium-iodate), 525 mg Se (as Sodium-selenite).
[3] Analyzed concentration of zinc in the basal diet without ZnO supplementation. The other diets contained 57 and 2425 mg/kg, respectively.

Table 5.4: Primer sequences, product length and annealing temperatures

Specificity (target gene)	Primer name	Sequence	Product length[bp]	Annealing temperature [°C]	Reference
E. coli/ Hafnia/ Shigella (16S rRNA)	Entero-F	GTTAATACCTTTGCTCATTGA	340	58	2
	Entero-R	ACCAGGGTATCTAATCCTGTT			
Enterobacteriaceae (rpoB)	EntqPCR3417f	GTBTCDCCRCGCAGRC	435	55	6
	EntqPCR3852r	TGCGYCTGGTRATCTA			
Clostridial-Cluster I (16S rRNA)	CI-F1	TACCHRAGGAGGAAGCCAC	231	63	5
	CI-R2	GTTCTTCCTAATCTCTACGCAT			
Clostridial-Cluster XIVa (16S rRNA)	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	60	4
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			
Clostridial-Cluster IV (16S rRNA)	sg-Clept-F	GCACAAGCAGTGGAGT	239	60	4
	sg-Clept-R	CTTCCTCCGTTTTGTCAA			
Bacteroides-Prevotella-Porphyromonas (16S rRNA)	BPP1	GGTGTCGGCTTAAGTGCCAT	140	55	1
	BPP2	CGGAYGTAAGGGCCGTGC			
Lactobacillus spp. (16S rRNA)	LAC-1	AGCAGTAGGGAATCTTCCA	341	58	1
	LAC-2	CACCGCTACACATGGAG			
Enterococcus spp. (16S rRNA)	Ent1	CCCTTATTGTTAGTTGCCATCATT	144	61	1
	Ent2	ACTCGTTGTACTTCCCATTGT			
Bifidobacterium spp. (16S rRNA)	g-BIFID-F	TCGCGTCYGGTGTGAAAG	243	58	1
	g-BIFID-R	CCACATCCAGCRTCCAC			
DGGE Eubacteria (rpoB)	New rpoB-DGGE (F)	TCA CGG TAA CAA RGG	431	50	7
	New rpoB-DGGE (R)	*AGT GCC CAT ACT TCC AT			
DGGE Enterobacteria (rpoB)	EntqPCR3417f	GTBTCDCCRCGCAGRC	435	50	6
	EntqPCR3852r	*TGCGYCTGGTRATCTA			

*= GC Clamp added: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGC

References

1 = Rinttilä et al., 2004; 2 = Malinen et al., 2003; 3 = Walter et al., 2000; 4 = Matsuki et al., 2004. 5 = Song et al., 2004; 6 = unpublished; developed in the Institute of Animal Nutrition, Freie Universtität Berlin; 7 = Perumbakkam et al., 2011

Table 5.5: Bacterial growth response to zinc [40 µg mL^{-1}and 20 µg mL^{-1}] supplemented medium in stomach and jejunum digesta samples of piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media).

		40 µg·mL^{-1} zinc						20 µg·mL^{-1} zinc					
		Stomach			Jejunum			Stomach			Jejunum		
Day	Time [h]	low Zn	high Zn	p-value	low Zn	high Zn	p-value	low Zn	high Zn	p-value	low Zn	high Zn	p-value
32	2	0.002	0.004	0.663	0.001	0.002	0.516	-0.001	-0.004	0.653	0.010	0.003	0. 346
	4	-0.018	-0.022	0.232	-0.005	-0.002	0.843	-0.015	-0.009	0.481	-0.008	-0.006	0.498
	6	-0.077	-0.042	0.072	-0.048	-0.020	0.044	-0.019	-0.016	0.387	-0.006	-0.011	0.178
	8	**-0.098^{a}**	**-0.071^{b}**	0.042	**-0.092^{a}**	**-0.015^{b}**	0.026	-0.022	-0.021	0.264	-0.019	-0.021	0.348
	10	**-0.131^{a}**	**-0.092^{b}**	0.028	-0.094	-0.064	0.079	-0.013	-0.011	0.587	-0.027	-0.025	0.751
	12	-0.110	-0.086	0.322	**-0.087^{a}**	**-0.057^{b}**	0.047	-0.005	-0.009	0.115	-0.032	-0.025	0.674
	14	-0.108	-0.073	0.107	**-0.085^{a}**	**-0.047^{b}**	0.014	0.008	0.010	0.425	-0.031	-0.022	0.379
	16	-0.075	-0.066	0.862	-0.042	-0.031	0.182	0.008	0.012	0.611	-0.022	-0.021	0.421
39	2	-0.005	-0.003	0.473	-0.004	-0.007	0.657	0.002	0.001	0.264	0.005	0.006	0.822
	4	-0.021	-0.007	0.182	-0.037	-0.011	0.483	-0.03	-0.007	0.814	-0.008	-0.011	0.735
	6	**-0.047**	**-0.010**	0.047	**-0.122^{a}**	**-0.074^{b}**	0.046	-0.058	-0.051	0.185	-0.026	-0.017	0.548
	8	-0.038	-0.019	0.062	**-0.136^{a}**	**-0.079^{b}**	0.041	-0.072	-0.082	0.648	-0.047	-0.037	0.199
	10	-0.046	-0.032	0.393	-0.091	-0.067	0.287	-0.068	-0.071	0.228	-0.062	-0.045	0.694
	12	-0.043	-0.039	0.744	-0.082	-0.045	0.154	-0.062	-0.057	0.194	-0.052	-0.033	0.281
	14	-0.038	-0.034	0.502	-0.066	-0.029	0.675	-0.021	-0.019	0.347	-0.041	-0.023	0.106
	16	-0.036	-0.023	0.199	0.025	0.030	0.722	-0.014	-0.008	0.365	-0.021	-0.029	0.371
46	2	0.008	0.007	0.283	-0.004	0.004	0.682	0.011	0.000	0.294	-0.003	-0.002	0.455
	4	-0.007	-0.012	0.736	-0.008	0.001	0.244	-0.001	-0.004	0.844	0.001	-0.004	0.627
	6	-0.020	-0.031	0.646	-0.049	-0.018	0.673	-0.017	-0.014	0.618	-0.009	-0.003	0.329
	8	-0.059	-0.070	0.209	-0.053	-0.016	0.541	-0.040	-0.034	0.529	-0.014	-0.013	0.405
	10	-0.078	-0.083	0.482	-0.046	-0.006	0.289	-0.029	-0.031	0.374	-0.014	-0.013	0.238
	12	-0.082	-0.073	0.833	-0.033	-0.006	0.415	-0.024	-0.023	0.845	-0.010	-0.011	0.424
	14	-0.071	-0.057	0.252	-0.021	-0.009	0.364	-0.014	-0.019	0.197	-0.010	-0.012	0.642
	16	-0.053	-0.043	0.713	0.014	0.012	0.475	-0.008	-0.011	0.649	-0.007	-0.017	0.112
53	2	0.002	0.002	0.773	-0.001	0.003	0.684	0.001	0.002	0.452	0.003	0.002	0.681
	4	-0.002	-0.007	0.824	-0.020	-0.013	0.519	-0.002	-0.009	0.501	-0.008	-0.002	0.509
	6	-0.018	-0.015	0.299	-0.048	-0.035	0.643	-0.005	-0.011	0.398	-0.012	-0.011	0.744
	8	-0.020	-0.005	0.658	-0.037	-0.026	0.511	-0.011	-0.012	0.475	-0.015	-0.016	0.298
	10	-0.010	-0.005	0.428	-0.012	-0.011	0.301	-0.020	-0.022	0.388	-0.001	-0.003	0.365
	12	-0.005	-0.003	0.606	0.007	0.015	0.284	-0.013	-0.014	0.479	0.001	0.003	0.714
	14	-0.001	0.001	0.241	0.011	0.021	0.512	-0.004	-0.005	0.624	0.005	0.006	0.542
	16	0.014	0.008	0.109	0.018	0.036	0.342	0.007	0.012	0.571	0.007	0.016	0.455

[a, b] Means within a row with different superscripts differ ($P < 0.05$). Significant differences ($P < 0.05$) between treatments are highlighted in bold.

Table 5.6: Cell numbers of bacterial groups in stomach samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide before and after 16h incubation

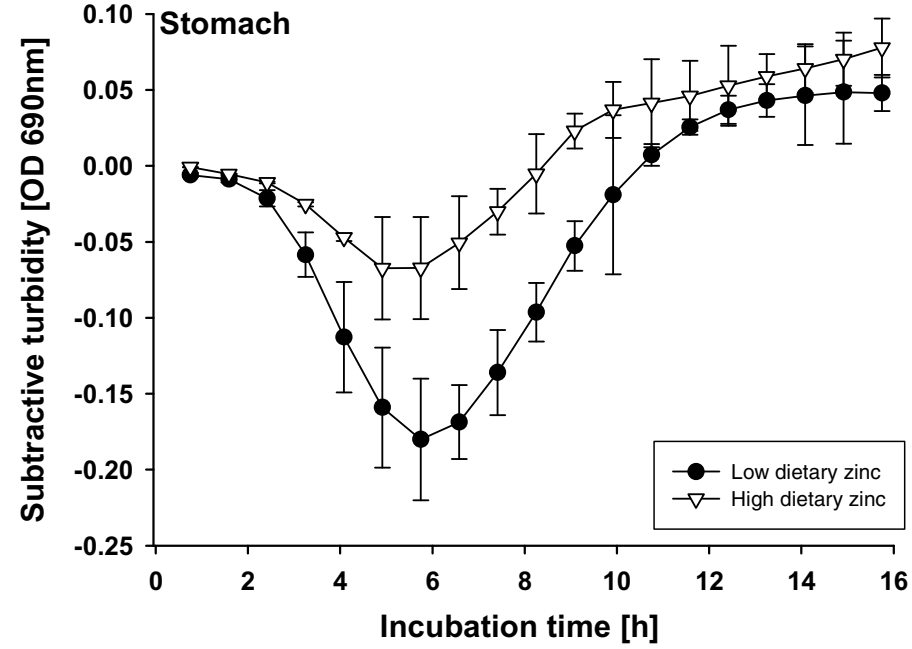
	Incubation time	0h		16h			
	Zinc in medium	-		0 µg/ml ZnO		80 µg/ml ZnO	
	Treatment group	low Zn	high Zn	low Zn	high Zn	low Zn	high Zn
32d	Bifidobacteria	6.7 ± (0.4)	5.9 ± (1.2)	9.2 ± (1.0)	9.2 ± (0.5)	9.1 ± (0.9)	9.1 ± (0.3)
	Bac.-Prevo.-Porphyromonas	6.4 ± (0.2)	6.1 ± (0.6)	4.9 ± (0.4)	5.2 ± (0.4)	4.9 ± (0.5)	5.4 ± (0.7)
	Clostridium Cluster I	4.9 ± (0.2)	5.8 ± (0.3)	6.8 ± (0.3)	7.2 ± (1.8)	7.2 ± (0.2)	7.1 ± (1.3)
	Clostridium Cluster XIVa	6.2 ± (1.1)	5.8 ± (0.1)	7.4 ± (0.2)	7.0 ± (0.2)	7.4 ± (0.3)	7.8 ± (0.3)
	Clostridium Cluster IV	4.1 ± (0.2)	4.6 ± (0.2)	4.8 ± (0.2)	4.7 ± (0.7)	4.9 ± (0.2)	5.0 ± (0.3)
	Enterobacteria	4.6 ± (0.5)	5.3 ± (0.1)	8.5 ± (0.1)	8.9 ± (1.1)	9.1 ± (0.4)	9.8 ± (0.6)
	Lactobacilli	7.0 ± (0.2)	7.4 ± (0.8)	10.3 ± (0.2)	10.4 ± (0.1)	9.7 ± (0.2)	9.7 ± (0.2)
	Enterococci	4.4 ± (0.1)	4.6 ± (0.2)	6.0 ± (0.4)	6.5 ± (1.5)	6.3 ± (0.5)	7.6 ± (2.7)
39d	Bifidobacteria	7.0 ± (0.4)	6.2 ± (1.1)	9.8 ± (0.3)	9.4 ± (0.6)	9.9 ± (0.4)	9.1 ± (0.8)
	Bac.-Prevo.-Porphyromonas	6.5 ± (0.1)	5.9 ± (0.9)	5.6 ± (0.1)	5.5 ± (0.5)	5.9 ± (0.1)	6.9 ± (0.5)
	Clostridium Cluster I	6.0 ± (0.4)	5.9 ± (0.5)	7.4 ± (0.1)	7.5 ± (0.2)	7.7 ± (0.2)	8.1 ± (0.1)
	Clostridium Cluster XIVa	7.7 ± (0.6)	6.1 ± (1.3)	7.3 ± (0.2)	7.2 ± (0.6)	7.5 ± (0.4)	7.7 ± (0.1)
	Clostridium Cluster IV	5.1 ± (0.1)	4.5 ± (0.5)	4.7 ± (0.6)	4.5 ± (0.7)	4.6 ± (0.5)	4.9 ± (0.2)
	Enterobacteria	5.6 ± (0.1)	5.6 ± (0.6)	8.6 ± (0.2)	9.1 ± (1.2)	9.1 ± (0.1)	9.8 ± (0.4)
	Lactobacilli	8.0 ± (0.2)	7.6 ± (0.2)	10.6 ± (0.1)	10.5 ± (0.1)	9.7 ± (0.1)	9.7 ± (0.3)
	Enterococci	5.1 ± (0.2)	5.1 ± (0.3)	6.0 ± (1.4)	6.5 ± (0.9)	7.0 ± (1.5)	6.7 ± (0.7)
46d	Bifidobacteria	4.9 ± (1.3)	5.3 ± (0.3)	8.4 ± (0.9)	8.0 ± (0.1)	8.1 ± (0.5)	8.2 ± (0.1)
	Bac.-Prevo.-Porphyromonas	5.6 ± (0.9)	6.1 ± (0.1)	5.1 ± (0.1)	5.8 ± (0.2)	5.3 ± (0.6)	6.7 ± (0.1)
	Clostridium Cluster I	4.0 ± (0.2)	4.7 ± (0.4)	6.1 ± (0.3)	7.1 ± (0.2)	6.7 ± (0.2)	7.6 ± (0.4)
	Clostridium Cluster XIVa	5.3 ± (0.4)	5.7 ± (0.3)	6.7 ± (0.2)	7.2 ± (0.3)	7.2 ± (0.6)	7.7 ± (0.1)
	Clostridium Cluster IV	3.9 ± (0.7)	4.5 ± (0.4)	6.0 ± (0.2)	5.3 ± (0.2)	5.8 ± (0.1)	5.2 ± (0.2)
	Enterobacteria	3.9 ± (0.1)	4.4 ± (0.1)	9.1 ± (1.0)	9.2 ± (0.5)	9.2 ± (0.7)	9.4 ± (0.3)
	Lactobacilli	5.7 ± (1.5)	5.1 ± (0.5)	10.1 ± (0.1)	10.3 ± (0)	9.1 ± (0.1)	9.6 ± (0.5)
	Enterococci	4.5 ± (0.1)	5.2 ± (0.6)	8.2 ± (0.7)	7.4 ± (1.4)	8.6 ± (0.8)	7.9 ± (1.4)

Table 5.7: Cell numbers of bacterial groups in jejunum samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide before and after 16h incubation

	Incubation time	0h		16h			
	Zinc in medium	-		0 µg/ml ZnO		80 µg/ml ZnO	
	Treatment group	low Zn	high Zn	low Zn	high Zn	low Zn	high Zn
32d	Bifidobacteria	6.7 ± (1.5)	5.5 ± (0.4)	10.1 ± (0.3)	8.9 ± (1.4)	9.3 ± (0.4)	8.5 ± (1.4)
	Bac.-Prevo.-Porphyromonas	5.8 ± (1.1)	5.2 ± (0.4)	4.9 ± (0.4)	3.6 ± (0.1)	5.0 ± (0.7)	4.6 ± (0.4)
	Clostridium Cluster I	5.0 ± (1.2)	5.5 ± (0.8)	8.3 ± (0.7)	8.1 ± (0.7)	8.2 ± (0.9)	8.2 ± (0.4)
	Clostridium Cluster XIVa	5.9 ± (0.5)	5.2 ± (0.1)	7.8 ± (1.0)	6.8 ± (0.2)	8.1 ± (0.5)	6.8 ± (0.1)
	Clostridium Cluster IV	4.1 ± (0.4)	4.2 ± (0.2)	4.1 ± (0.2)	4.7 ± (0.1)	4.9 ± (0.1)	4.4 ± (0.6)
	Enterobacteria	4.8 ± (0.5)	5.3 ± (1.8)	8.7 ± (0.1)	9.7 ± (0.9)	9.1 ± (0.3)	9.8 ± (0.4)
	Lactobacilli	7.1 ± (0.4)	6.2 ± (1.2)	10.2 ± (0.1)	10.3 ± (0)	9.6 ± (0.5)	9.7 ± (0.1)
	Enterococci	2.5 ± (0.4)	2.8 ± (0.9)	6.3 ± (1.6)	7.1 ± (2.8)	6.8 ± (1.3)	7.5 ± (3.4)
39d	Bifidobacteria	5.5 ± (0.6)	6.4 ± (0.1)	10.4 ± (0.5)	9.4 ± (0.9)	10.3 ± (0.1)	9.2 ± (1.0)
	Bac.-Prevo.-Porphyromonas	5.9 ± (0.4)	6.0 ± (0.1)	5.0 ± (0.2)	5.7 ± (0.1)	5.7 ± (0.1)	5.9 ± (0.2)
	Clostridium Cluster I	5.9 ± (0.9)	6.5 ± (0.3)	7.3 ± (0.1)	7.5 ± (0.5)	8.0 ± (0.3)	8.0 ± (0.1)
	Clostridium Cluster XIVa	5.9 ± (0.4)	6.0 ± (0.3)	7.6 ± (0.1)	7.1 ± (0.2)	7.8 ± (0.1)	7.6 ± (0.1)
	Clostridium Cluster IV	4.0 ± (0.5)	3.6 ± (0.1)	4.8 ± (0.1)	3.6 ± (0.7)	4.3 ± (0.5)	4.5 ± (0.5)
	Enterobacteria	5.8 ± (0.4)	5.6 ± (0.2)	9.5 ± (0.3)	9.6 ± (0.2)	10.1 ± (0.2)	9.9 ± (0.3)
	Lactobacilli	7.6 ± (0.3)	6.9 ± (0.1)	9.9 ± (0.6)	10.4 ± (0.1)	10.1 ± (0.3)	10.0 ± (0.6)
	Enterococci	3.0 ± (0.4)	3.0 ± (0.1)	6.3 ± (0.5)	6.1 ± (1.5)	6.6 ± (0.7)	7.2 ± (0.4)
46d	Bifidobacteria	4.6 ± (1.2)	4.1 ± (0.4)	8.6 ± (0.2)	7.7 ± (0.5)	8.4 ± (0.1)	8.0 ± (0.4)
	Bac.-Prevo.-Porphyromonas	5.3 ± (0.1)	4.8 ± (0.3)	5.6 ± (0.1)	5.4 ± (0.1)	5.8 ± (0.7)	6.0 ± (0.6)
	Clostridium Cluster I	3.9 ± (0.5)	4.9 ± (0.2)	5.9 ± (0.3)	6.9 ± (0.2)	6.9 ± (1.0)	7.6 ± (0.3)
	Clostridium Cluster XIVa	5.0 ± (0.2)	5.0 ± (0.1)	6.7 ± (0.1)	6.8 ± (0.1)	7.0 ± (0.2)	7.1 ± (0.1)
	Clostridium Cluster IV	3.7 ± (0.1)	3.8 ± (0.3)	4.7 ± (1.7)	5.5 ± (0.7)	4.5 ± (1.7)	4.9 ± (0.1)
	Enterobacteria	3.5 ± (0.2)	4.0 ± (0.4)	9.0 ± (0.8)	9.2 ± (0.3)	9.6 ± (1.7)	9.9 ± (0.4)
	Lactobacilli	5.5 ± (2.1)	4.1 ± (0.3)	9.8 ± (0.4)	9.3 ± (0.5)	9.0 ± (0.5)	8.7 ± (0.7)
	Enterococci	3.3 ± (0.5)	3.9 ± (0.1)	7.8 ± (0.3)	7.6 ± (1.0)	8.0 ± (0.2)	7.9 ± (1.3)

Table 5.8: Diversity indices for the enterobacterial composition after 16h incubation of stomach and jejunum samples of piglets fed diets containing 57 ppm (low Zn) or 2425 ppm (high Zn) dietary zinc oxide in media supplemented with 0 or 80 µg mL^{-1} ZnO

	Stomach				Jejunum			
	0 µg Zn		80 µg Zn		0 µg Zn		80 µg Zn	
	low Zn	high Zn	low Zn	high Zn	low Zn	high Zn	low Zn	high Zn
32d								
Richness	8	9	8	6	9	6	7	10
Shannon	2.82	3.07	2.79	2.13	3.09	2.05	2.64	3.36
Evenness	0.835	0.84	0.834	0.819	0.841	0.815	0.835	0.846
39d								
Richness	8	6	6	9	6	9	9	8
Shannon	2.74	2.17	1.83	2.92	2.14	3.17	3.22	2.76
Evenness	0.832	0.821	0.802	0.833	0.819	0.844	0.847	0.833
46d								
Richness	4	9	8	9	5	8	9	9
Shannon	1.36	3.36	2.84	3.31	1.71	2.96	2.79	2.95
Evenness	0.798	0.853	0.837	0.851	0.806	0.843	0.827	0.834



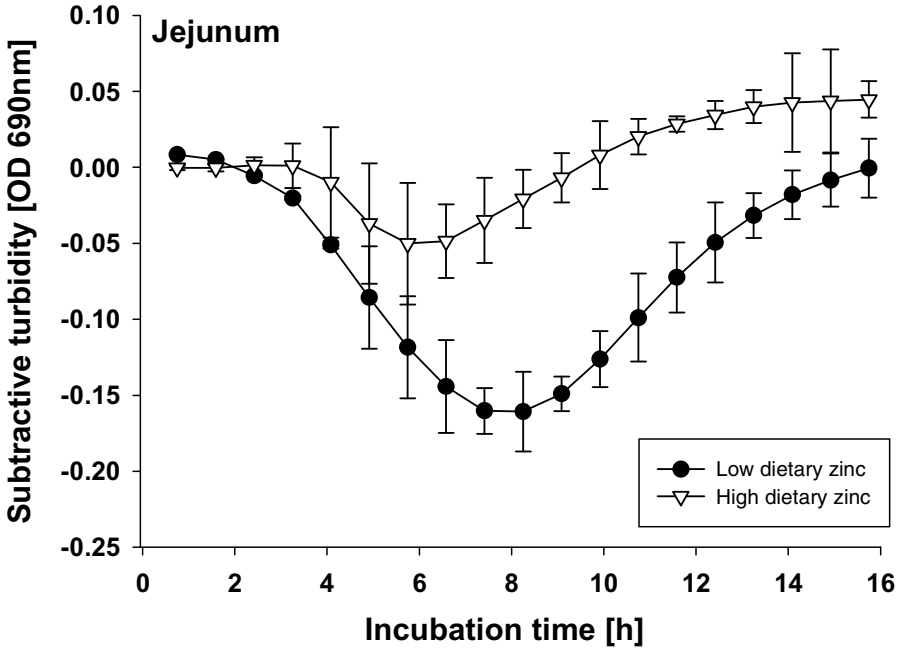


Figure 5.3: Detailed display of the bacterial growth response to zinc [80 µg mL^{-1}] supplemented medium in digesta samples of 35d old piglets fed low or high dietary zinc (data after subtraction of turbidity in non-zinc supplemented media)

5.8 Author Contributions

Conceived and designed the experiments: JZ WV. Performed the experiments: ICS. Analysed the data: WV. Wrote the paper: ICS WV JZ.

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Chapter 6. General discussion and conclusions

6.1 Discussion

Introduction

The gut microbiome is an integral part of the GIT in piglets and interacts in many diverse ways with the host in terms of gut function and animal health [5, 6, 180]. There are many factors influencing the microbial composition in farm animals like initial contact with bacteria, the host immune system or environmental stress. Particularly weaning is a stressful situation for piglets and can cause serious diseases leading to animal losses and therefore economic problems as reviewed in **Chapter 2**. To overcome the problems of this challenging time period, feed additives that aim to modify the intestinal microbiota such as zinc are more and more in use. Studies have shown that zinc interferes with the intestinal microbiota in farm animals and has the potential to improve animal performance and health when it is used in high amounts [9]. In so called pharmacological doses it has been shown that ZnO acts bactericidal. However, there are only a few studies on the influence on the microbial communities. It could be shown in a study by Hojberg et al. that lactobacilli counts were reduced and coliforms were increased, but enterococci were not influenced [142]. The results are consistent with studies from Mores (1998) [146], who found that ZnO supplementation was effective in control of *E. coli* diarrhea but without reducing *E. coli* and Broom et al. (2006) [143], who found that ZnO reduces lactic acid bacteria and anaerobes, but *E. coli* was not affected.

However, the mode of action of ZnO as feed additive in young piglets is still unclear. Besides that, it is also necessary to investigate the optimal administration period to achieve maximal efficacy at a minimum of environmental damage. Furthermore, different effects may occur within the animal depending on dose and duration of the application.

In this chapter the results of both experimental approaches of the zinc trial will be combined and discussed to provide a comprehensive view on the ZnO administration and its effect on the pig microbiome.

Summary of the experiments

In the first study, 17 different primer sets were used to investigate the bacterial composition between the different zinc treatment groups via qPCR. The primers were chosen to cover the known dominating bacterial groups and species on the pig GIT. The quantitative measurement of bacterial cell numbers via qPCR is commonly known to have some drawbacks including PCR bias, enzymatic PCR inhibition and insufficient artificial calibration methods [181]. The quality and quantity of this method is also depending on bacterial cell lysis and DNA extraction efficiency [182]. To minimize these factors different extraction protocols were validated and a calibration method based on spiked samples of known amounts of bacterial cells to overcome DNA extraction differences was used [183]. The advantage of qPCR approaches is the high sample throughput combined with high specific detection accuracy which makes this method an adequate instrument for quantifying selected bacterial genera and species [181].

In the second study, digesta samples from the stomach and jejunum were incubated in media containing different zinc concentrations to estimate changes in bacterial growth kinetics. To assess lag time, specific growth rate and maximum growth, bacteria were incubated in a growth medium. It is known that artificial environments lead to a shift in the bacterial composition for organisms favoring the compounds in the media. It is also not possible to assess the bacterial composition only by measuring the growth kinetics. Furthermore, without additional analysis, it remains unknown which types of bacteria gained growth advantage or were suppressed by zinc in this artificial system. However, to overcome these disadvantages a medium was chosen to support the growth of a wide variety of bacteria. Also, anaerobic experimental conditions were chosen to minimize growth bias. Additionally, qPCR assays were used for samples during bacterial growth to monitor the most abundant bacteria known for the gut ecosystem. Therefore the *ex vivo* trial could give insights into growth kinetics under different zinc concentrations as well as adaptation and resistance mechanisms of selected bacterial groups.

Influence on bacterial composition

The major known bacterial groups in the pig GI tract include *Lactobacillus, Enterococcus, Streptococcus, Bacteroides, Prevotella, Clostridium , Bifidobacterium* and enterobacteria [1, 26, 47]. In general, lactic acid bacteria are the dominant functional bacterial group in the

upper GIT. In this study we put our emphasis on lactobacilli and other important lactic acid bacteria, but also on enterobacteria and clostridia which are known to harbor some pig relevant pathogens.

Impact of ZnO on lactobacilli

According to our observations, the lactobacilli and three of five examined *Lactobacillus* species responded to high dietary ZnO with reduced cell numbers throughout the trial period. Among the lactobacilli, *L. amylovorus* and *L. reuteri* are found to be the dominating species in the small intestine [147, 184]. The most prevalent detected species in this study was *L. amylovorus*, but the even higher cell numbers of *Lactobacillus* spp. as a group suggests that there were other *Lactobacillus* species not examined in this study.

L. johnsonii and *L. reuteri* only showed significantly reduced cell numbers in the high dietary ZnO group on the 32nd day of life. The most drastic differences were observed in the small intestine (jejunum, ileum) and in the first sampling week. Therefore, it remains unclear whether and how many *Lactobacillus* species are in fact influenced by the zinc treatment. This coincides with similar studies with ZnO used as feed additive with piglets [142, 143, 146]. The results suggest that the influence ZnO acts individually different even from species to species within the lactobacilli group. This could also be shown by a study from Liedtke and Vahjen (2012) [151], where a broad range of different species were tested according to their zinc resistance in an *in vitro* study. In that study the authors could show that the zinc resistance of *L. amylovorus* was lower compared to other *Lactobacillus* species or species from the enterobacterial group. Interestingly, *L. reuteri* and *L. johnsonii* showed reduced cell numbers only at the first days of the trial and seem to recover after a two week administration of ZnO. This was not the case for *L. amylovorus,* as this species showed consistently lower cell numbers throughout the trial due to high zinc treatment. This again emphasizes an individual response and resistance against heavy metals among bacteria within one bacterial group.

The impact of ZnO on enterobacteria

The enterobacteria, which are commonly assumed as being the antagonists of lactic acid bacteria were only decreased at the first sampling day (d 32), but after two weeks enterobacteria numbers remained stable. As only two group specific primers was used for

analyzing enterobacteria, it is unknown which species were affected by the zinc treatment and whether some species were more resistant or not. However, a qPCR assay for the phylogenetic *Escherichia/ Hafnia/ Shigella* group showed that differences were less pronounced than for the total enterobacteria group.

In Gram-negative bacteria, heavy metal resistance mechanisms are known already [162]. Effective influx and efflux transport systems are well developed in enterobacteria. This bacterial group also requires iron as an essential trace element [185]. Transport systems for divalent ions (efflux and influx) are therefore very important for enterobacteria and may also be responsible for an increased zinc resistance by helping enterobacteria to adapt to higher heavy metal concentrations in the environment. It has also been perceived that Gram-negative bacteria have specific proton-cation antiporter efflux systems for heavy metals, while Gram-positive bacteria primarily use P-type efflux ATPases to pump zinc out of their cells [186]. Furthermore, genes for proteins of the Resistance-nodulation-cell-division transporter protein family, specifically for the heavy-metal efflux protein family, are much more abundant in Gram-negative bacteria. Therefore, heavy metal resistance mechanisms in general are more common and they seem to be more efficient in Gram-negative bacteria. As a consequence, one could speculate that enterobacteria possess more efficient systems to expel intracellular zinc [187] than the Gram-positive lactobacilli.

Furthermore, horizontal gene transfer is well described for Gram-negative bacteria [188] and may also be a reason for efficient and fast adaption to different environments. Knowledge of plasmid exchange in Gram-positive microorganisms however is still limited [189].

A study could show in a pyrosequencing approach that the diversity of enterobacteria increased after zinc treatment [149]. This could indicate that the zinc treatment may replace previously dominant coliforms and more resistant enterobacteria are able to occupy new niches, possibly by enhanced gene transfer. Other studies could also show either no influence [143], an increase of enterobacteria [142] or even an increased phenotype stability of coliform isolates after the weaning process [145]. These studies further illustrate that enterobacteria may in general have a higher capability to deal with high dietary ZnO.

Results of this study challenge the classic model of harmful enterobacteria vs. beneficial lactic acid bacteria. Under the conditions of the feeding trial, animal performance and health were not impaired negatively throughout the whole trial [190], although lactobacilli were reduced and enterobacterial diversity increased.

Another important consideration is that an increased colonization potential and possibly increased diversity of enterobacteria due to high intestinal zinc concentrations may also have a negative impact on the colonization potential of pathogenic *E. coli* strains. *E. coli* induced diarrhea is often reduced in piglets fed high dietary zinc diets and a few studies examined the influence of ZnO on *E. coli* challenged piglets [191-193]. It was shown for example, that piglets challenged with a controlled dose of enterotoxigenic *E. coli* (ETEC) K88 and fed a ZnO-supplemented diet recovered more rapidly than controls in terms of both diarrhea and ETEC shedding [192]. Experiments on cultured cells could show that ZnO reduces K88 fimbriae adhesion to the intestinal epithelial cells [158]. From an ecological point of view it is important to emphasize that pathogenic *E. coli* strains may encounter a higher intra-group competition due to increased diversity of enterobacteria caused by the zinc treatment. This selection pressure may lead to higher resistance to zinc or to bacteriocin production of closed related species which brings up a high competitive situation for spatial distribution and therefore a major disadvantage for "newcomers" to colonize the porcine small intestine.

Impact of dietary ZnO on other bacterial groups

Weissella spp. and *Leuconostoc* spp., which also belong to the lactic acid bacteria, were not reduced as strongly as other lactobacilli in this study, in contrast to a similar study in older animals (56d old) [149] in which it was demonstrated that *Weissella* spp. and *Leuconostoc* spp. sequence reads were increased significantly due to high dietary zinc. This could either reflect an individual response to the feed additive or may indicate a competition advantage due to a reduction of other lactobacilli in the gut ecosystem.

Streptococci, bifidobacteria and enterococci cell counts stayed stable throughout the whole trial and were therefore more resistant against high dietary zinc. This is in agreement with a work from Hojberg (2005), where enterococci were even increased significantly in zinc treated animals [142].

The class Clostridia includes diverse bacteria both of medical and environmental importance. Three different clostridial clusters were analyzed in this study. Cluster I is the largest group of *Clostridia* and contains some facultative pathogens like *Clostridium perfringens*. Cluster IV and cluster XIVa contain many proteolytic and cellulolytic clostridia responsible for many fermentation processes in the gut [194]. The cell numbers of all studied clostridial clusters seemed to be unaffected by high dietary zinc concentrations and their highest abundance

could be found in the hind gut. In the zinc treated animals on day 42, the clostridium cluster IV was reduced in the jejunum and ileum, but cell numbers stayed stable at later time points which may indicate an adaptation skill after a certain time. The large Gram-negative *Bacteroides-Prevotella-Porphyromonas* cluster, one of the predominant bacterial groups in the gut, was also investigated, but no significant difference between the treatment groups could be found.

Both, *Clostridia* and *Bacteroidetes* are strict anaerobic and attain high cell numbers predominantly in the large intestine participating in fermentation processes and short chain fatty acid production. They also seem to play an important role in the small intestine in weaned piglets, where they could be detected in lower cell numbers. In the combined Spearman correlation analysis done in this study, the clostridial cluster XIVa exhibited a negative correlation to zinc ions in the small intestine like most studied bacterial groups, but a positive correlation in the colon. Together with the high cell numbers in the colon these clostridia may have gained an additional colonization advantage in the colon during the development of the microbiota. This again could be due to better zinc resistance strategies of certain species gaining a colonizing advantage.

The *Bacteroides-Prevotella-Porphyromonas* cluster showed positive correlations to free zinc in the small intestine but the cell numbers of this group was not affected significantly by ZnO treatment. The reduction of the dominating lactobacilli by ZnO may create more niches leading to a replacement of dominant bacteria by other bacterial groups like the *Bacteroides-Prevotella-Porphyromonas* group.

Comparison with *ex vivo* analyses

In general, the *ex vivo* experiments showed that growth occurred even at the highest zinc concentration (80 µg zinc ml^{-1} medium), so nutrient composition must have been sufficient to support bacterial growth for some bacterial groups.

The data from growth kinetics showed a lower initial bacterial growth (longer lag time) in samples from the digesta from animals fed the high dietary zinc. As the lag time in a growth curve relates directly to the physiological fitness of a bacterial population, lower initial growth confirms the bacteriostatic effect of dietary ZnO in piglets. It can also be assumed that the higher initial growth depression of the bacteria of the high dietary zinc group arises from a higher physiological stress compared to the bacteria in digesta samples of the low dietary zinc

group. At the end of the incubation we could mostly observe a higher maximum OD (optical density) in the zinc supplemented media which also could indicate a competitive success of zinc resistant species. These species, already preselected in the digesta sample of the high zinc group for high zinc levels will thus outgrow zinc sensitive microorganisms in the complex medium yielding higher final turbidity.

However, higher maximum OD values were mostly observed in younger animals from samples of the high dietary zinc group and although the numerical trend of a higher turbidity continued in samples from day 46 and day 53, significant differences could only be found in younger animals. The specific growth rates (slope of exponential growth) also showed the same trend. Significantly higher specific growth was often observed in digesta samples of the high dietary group from the first two weeks of the trial. One possible explanation could lie in population dynamic processes between nutrient competition and zinc resistance advantage. In samples from young animals both zinc resistant and non-resistant bacteria should be evenly distributed and are both types are challenged in high zinc media. This leads to a fitness advantage for high zinc resistant bacteria, consequently leading to a high specific growth rate. However, in samples from older animals the bacterial community may already be dominated by zinc resistant microorganisms, which leads to a higher competition only for nutrient in the complex media. It was not possible to assess all types of bacteria which gained growth advantage or were suppressed by zinc in this artificial system but the dominating bacterial groups and species were monitored via qPCR.

According to the qPCR data, lactic acid bacteria as well as enterobacteria were not particularly sensitive to ZnO neither from samples of the high zinc group nor the low zinc group. However, according to the measured cell number of lactobacilli, the increase for lactobacilli was much less pronounced than for enterobacteria. It could therefore be speculated that a reduction of dominant *Lactobacillus* species occurred, which would leave an increased amount of substrates in the media for zinc resistant lactic acid bacteria or other bacteria. In addition, as the DGGE analysis has shown an increased diversity of enterobacteria after growth, it is likely that enterobacteria gained a growth advantage under high zinc conditions.

The data from qPCR shows also that the *Bacteroides-Prevotella-Porphyromonas* cluster, Clostridium cluster IV and cluster XIVa did not grow well in the employed *ex vivo* system.

Here, it is also expected that the competition during the incubation in the media led to lower cell numbers.
However, it can be assumed that in the digesta samples taken from the stomach and proximal small intestine the dominant microbiota consists of lactic acid bacteria and enterobacteria.

Conclusions on the impact of ZnO on bacterial composition and *ex vivo* growth

In this study a range of bacterial groups and species were monitored whether high doses of dietary ZnO have an influence on the gut microbial composition. Overall it could be observed that the cell numbers of certain *Lactobacillus* species were reduced mainly in the stomach and small intestine. Enterobacteria were reduced only in the first two weeks after weaning. The reduction of dominant bacteria may give rise to increased colonization by other bacterial groups or species, because additional colonization space is available. This “niche concept” has been shown for other environments also [195]. It also can be concluded that in general dominating and highly abundant bacteria may compensate systematic disturbances via reproduction or cell number whereas less numerous species could adapt by investing more in resistance mechanisms. These different selection strategies could already be shown in soil bacteria [196]. ZnO leads also to general bacterial growth depression, but bacterial adaptation to zinc occurs within 2 to 3 weeks. Due to the fact that the gut ecosystem seems to be in constant flow equilibrium, diminished groups of organisms may be replaced by more adapted species which must not lead cogently to a lower stability of the bacterial communities. It could also been shown that the diversity of certain bacterial groups could be increased by reduction of dominant organisms and it can be concluded that previously rare bacteria can establish their colonization potential and occupy new niches.

Bacterial Metabolites

Beside the assessment of the influence of high dietary zinc on the bacterial community structure in the pig gut, it was the purpose of this thesis to highlight also some functional aspects. Microbial metabolites are important markers, which can be beneficial for the host [58]. According to the amount and abundance of certain metabolites combined with data from the bacterial communities it is possible to estimate functional aspects under influence of different treatments.

Analysis of the total concentration of bacterial metabolites showed reduced values in all animals along every intestinal segment after the zinc treatment. This may be caused by the general reduction of many bacterial groups especially lactobacilli and therefore a reduced net production of metabolites due to high dietary zinc. The strongest decrease could be found for propionate concentrations (significantly so on day 49 and 56) and also in n-butyrate concentrations, while acetate concentrations remained unchanged. In general, lactate serves as substrate for propionate production, but also for n-butyrate production in the intestinal tract [107]. Lactate, the main metabolite produced by lactic acid bacteria, was generally reduced in this study. Lactate is an important substrate for lactate utilizing bacteria such as *Megasphaera, Selenomonas,* and *Veillonella* [107] which convert lactate to acetate, propionate and butyrate. Propionate concentrations were also reduced in the small intestine. *Veillonella* spp. and *Selenomonas* spp. are known for strong lactate fermentation and may therefore also be influenced indirectly by reduced lactic acid bacteria [197, 198]. *Megasphaera elsdenii* converts lactate into butyrate [199], which was also reduced along all sampling sites. It can be concluded that many strict anaerobic bacteria also using lactate as energy source may therefore be limited due to reduced substrate input in the small intestine.

However, the interaction between lactate producing and lactate utilizing bacteria in the intestine of pigs is not well studied yet. The use of lactate as substrate in the gut is of course dependent on lactate production and a reduced lactate concentration would reduce any metabolic cross-feeding. Lower lactate concentrations could directly lead to reduced acetate, propionate and butyrate concentrations and may therefore also have an impact on hind gut microbiota as well as on host energy extraction. The hind gut microbiota could be modified due to reduced small intestinal input of fermentable carbohydrates or respective metabolites and a different microbial composition in the proximal GIT.

Interestingly, the total metabolite concentration was reduced through the whole trial period but this does not correlate well with bacterial cell numbers. Although the ZnO application is presumed to act bactericidal, the cell numbers seem to recover after two weeks, whereas the metabolite concentration was still negatively influenced. This may be due to undetected bacteria within a changed composition, which may be responsible for a different reduced metabolome. However, another reason could be the investment of the bacterial cell in energy consuming zinc resistance mechanisms, which would lead to a bacterial growth retardation resulting in lower cell numbers due to increased time for cell division. Therefore, ZnO may

not always act bactericidal, but could in fact only be a bacteriostatic agent, reducing bacterial metabolism without killing the bacterial cells.

Ecological considerations

In this study we could show an age dependent diminishing effect of zinc on bacterial growth and an increased degree of resistance in the high dietary zinc group. We propose that an adaptation of the intestinal microbiota occurred during the feeding trial. A similar observation could be made in different microbial ecosystems such as the soil environment [195]. The fingerprint analysis by DGGE depicts a strong clustering between zinc treatment groups and non-treatment groups, which might be proof for similar mechanisms influencing and changing bacterial patterns and communities in the samples. Diversity index calculations also showed that the high zinc treatment led to a higher diversity within the enterobacterial community. This has also been shown in a deep sequencing study [147]. This could be due to an individual capability of different species in dealing with different amounts of ZnO, but could also indicate an increased gene transfer, as many heavy metal resistance genes of enterobacteria are encoded on plasmids [200]. It is further possible that reduced lactic acid bacteria and hence less lactate production could form better colonizing conditions for enterobacteria. In the *ex vivo* trial we could furthermore show the strong capability of bacteria to adapt to changed environmental conditions, depending on time, concentration and duration of zinc administration. This correlates with *in vivo* results, as the highest differences between high and low dietary zinc treatments were observed after the first week after weaning.

It is interesting that the major effect of the feed additive was to reduce commensal lactobacilli, which are considered important for stabilizing the porcine gut ecosystem [35]. In this case the paradigm of the antagonistic nature of lactic acid bacteria against coliforms must be reconsidered. An increased number and diversity may also provide a more and more challenging environment for pathogenic bacteria and therefore be beneficial for the host [158, 192]. The positive correlation of the strict anaerobe *Bacteroides-Prevotella-Porphyromonas* group with free inorganic zinc especially in the jejunum may be founded in the reduction of lactobacilli, which are usually dominant in the small intestine. Therefore Bacteroidales may play a more important role in the small intestine as initially thought. Also clostridial cluster XIVa has shown a positive correlation with inorganic zinc but only in the colon, which may be an indication of a colonization advantage due to reduced bacterial cells of other groups.

Some Gram-positive bacterial groups like enterococci, streptococci or bifidobacteria were not influenced by the zinc treatment which may be founded in higher zinc resistance ability or utilization of freed niches due to a reduction of lactobacilli. Other bacterial groups showed an adaptation to ZnO which leads to the question whether a certain duration of administration should be considered. A long application of high doses zinc could have negative effects on the environment [201, 202] or can lead to bacterial zinc resistance jeopardizing the pharmacological efficacy for future applications. It could also be shown that zinc in animal farming might provoke antibiotic resistance [203, 204]. Bednorz et al. (2013) [205] could show that supplementation of zinc as a feed additive increases the amount of multi-resistant *E. coli*. The authors concluded that zinc used in long terms might promote the spread of antibiotic resistance in farm animals. By reducing the total bacterial number in the hind gut and diminishing bacterial metabolites, ZnO may also reduce the proportion of SCFAs providing the pig with energy [206]. This could lead to reduced performance in older animals. However, this study has elucidated the basic mode of action of ZnO on the intestinal microbiota, but some functional aspects remain unsolved.

6.2 Conclusions

The weaning period of piglets is the most critical part in animal production. It forces the animal into an extreme stress situation involving new environmental as well as social situations and most importantly, the adaptation of the GIT to solid feed. This often results in lower feed intake, a weakened immune system and changed gut physiology. Consequently the animals are prone to intestinal diseases like diarrhea caused by an imbalanced microbiota. Therefore, it is necessary to find solutions to stabilize the microbiota of the GIT of weaning piglets. In our study we could show that zinc acted as a powerful microbiome modifier, which affected certain bacterial groups transiently (enterobacteria) or permanently (lactobacilli).

With two experimental approaches the influence of dietary ZnO on structure and functional aspects of the intestinal microbiota in weaning piglets with molecular biological methods was studied. In summary, it can be stated that by the use of ZnO as feed additive in high doses the cell numbers of a range of bacterial groups were reduced mainly in the small intestine in the first two weeks after weaning. The most drastic influence could be found for lactobacilli and enterobacteria. It can be concluded that the changed environment leads to a competitive shift in bacterial communities through mechanisms of zinc resistance and spatial replacement due to loss of usually dominant bacteria. The *ex vivo* approach also showed that ZnO leads to a bacterial growth depression, but ZnO seems to act rather bacteriostatic than bacteriocidal. Adaptation to zinc occurred earlier in animals of the high dietary zinc treatment, but older animals from the low dietary zinc group also showed high adaptation ability to high zinc doses.

With this study we could show some interesting mechanisms of dietary ZnO with respect to intestinal bacterial communities. Further studies are required to improve our knowledge regarding other substances used as feed additives in animal nutrition associated with animal health.

Chapter 7. Summary/Zusammenfassung

Summary

Title of the PhD thesis: Influence of High Dietary Zinc on Structure and Selected Functional Aspects of Intestinal Microbial Communities in Piglets

The development of new feeding strategies to maintain gut health in newly-weaned pigs, in order to minimize the use of antimicrobial compounds such as antibiotic growth promoters has become essential to avoid the emergence of antibiotic resistant bacteria. Since the late 1980s, the use of pharmacological concentrations of zinc oxide (ZnO) was studied to prevent diarrhea and increased growth rates in weaning piglets (**Chapter 1**).

In **Chapter 2** a summary of the literature on gut microbiota – host interactions and zinc as feed additive is given. The main objective of this thesis was to investigate the pig gut microbiota under the influence of high dietary ZnO to improve our knowledge for structural and functional aspects of gut microbial communities and to estimate new feeding strategies to substitute antibiotic growth promoters. Detailed information on the study aims can be found in **Chapter 3**.

The two different experimental approaches (**Chapter 4 & 5**) used in this thesis are based on the same animal trial where different diets containing high and low amounts of ZnO on newly-weaned piglets are tested. Briefly, piglets were fed diets containing 57 (low) or 2425 (high) mg kg^{-1} zinc from analytical grade ZnO. For the first experiment (**Chapter 4**) intestinal contents from stomach, jejunum, ileum and colon were sampled from 32, 39, 46 and 53d old piglets and analyzed for bacterial cell numbers and main bacterial metabolites. The most drastic effects of high dietary zinc could be found one week after weaning in the stomach and small intestine. Cell numbers of Enterobacteriaceae, the *Escherichia* group as well as from *Lactobacillus* spp. and three abundant *Lactobacillus* spp. were reduced. The influence of high dietary zinc was transient for enterobacteria but permanent for *Lactobacillus* spp. No impact could be observed at cell numbers of bifidobacteria, enterococci, streptococci, *Weissella* spp. and *Leuconostoc* spp. as well as the *Bacteroides-Prevotella-Porphyromonas* group.

Among the microbial metabolites molar acetate ratios increased and propionate decreased in the proximal intestine and lower lactate concentrations were observed in the high dietary zinc

group throughout the feeding trial. However, the differences between the dietary groups in cell numbers and in microbial metabolites diminished in older animals.

For the second experiment (**Chapter 5**) digesta samples from the stomach and jejunum of 32, 39, 46 and 53d old animals (n = 6 per group) were sampled and incubated for 16h under anaerobic conditions in a complex media containing 80, 40, 20 and 0 µg mL^{-1} soluble Zink. Specific growth rate, maximum growth and lag time were calculated according to the obtained growth curves. The highest rate of growth and lowest lag times could be observed in the samples from animals fed the low dietary zinc concentration in media without added zinc. The samples from animals fed the high dietary zinc concentration showed highest growth rates and lowest lag time in the media supplemented with zinc. Bacterial growth of digesta samples from the high dietary zinc group was less influenced by zinc and recovered growth more rapidly than in the low dietary zinc group and samples from older animals of the low dietary zinc group also showed reduced growth depression. Bacterial cell number analysis from PCR results showed that lactobacilli were reduced by the zinc treatment, while bifidobacteria and enterococci showed increased growth in samples from the high dietary zinc treatment. Enterobacteria from samples of the high dietary zinc group showed an increased growth rate at high zinc concentrations in the medium. No differences were observed for the analyzed clostridial cluster and the *Bacteroides-Prevotella-Porphyromonas* group.

The studies have shown that the supplementation of feed with high doses of zinc leads to a reduced *ex vivo* - bacterial growth rate of bacteria from the stomach and jejunum and to transient and lasting effects during the development of the intestinal microbiota along the whole GIT *in vivo*, affecting composition as well as metabolic activity of weaned piglets. In view of the rapid bacterial adaptation to dietary zinc *ex vivo* as well as *in vivo* , the administration of ZnO as feed additive for weaned piglets might only be beneficial in a short period after weaning.

In **Chapter 6** the results of both experimental approaches are discussed and summarized with contributions to evaluate new feeding strategies in modern animal nutrition in respect of microbial communities in the gut.

Zusammenfassung

Dissertation zum Thema: Einfluss von pharmakologisch verabreichtem Zinkoxid auf Struktur und ausgewählte funktionelle Aspekte der intestinalen Mikrobiota im Absetzferkel

Die Entwicklung neuer Fütterungsstrategien zum Erhalt der Darmgesundheit im Absetzferkel gewinnt im Hinblick auf die Vermeidung von Antibiotika und das damit mögliche Aufkommen antibiotikaresistenter Bakterien mehr und mehr an Bedeutung. Seit Ende der 80'er Jahre wird Zinkoxid (ZnO) in erhöhter Dosierung als Futterzusatzstoff für das Absetzferkel untersucht (Überblick in **Kapitel 1**).

In **Kapitel 2** wird eine Übersicht auf forschungsrelevante Veröffentlichungen zum Thema Darmmikrobiota – Wirtsinteraktionen und Zinkverabreichung in pharmakologischen Dosen gegeben. Ziel der vorliegenden Dissertation war es, die Darmmikrobiota im Absetzferkel unter Einfluss von hochdosiertem ZnO als Nahrungszusatzstoff zu untersuchen. Im Vordergrund steht dabei der Wissenserhalt zu strukturellen und funktionellen Veränderungen von Darmbakterien um neue Fütterungsstrategien einzuschätzen beziehungsweise den Einsatz von Antibiotika als Wachstumsförderer zu vermindern. Eine detaillierte Übersicht zu diesem Thema ist in **Kapitel 3** zu finden. Den beiden experimentellen Ansätzen, beschrieben in **Kapitel 4 & 5**, liegt ein Tierversuch, in dem zwei unterschiedliche Diäten mit jeweils hohen und niedrigen Zinkdosen an Absetzferkel verabreicht wurden. Dabei wurden Landrassenferkel jeweils 57 (niedrig) oder 2425 (hoch) mg kg^{-1} ZnO (p.a. Qualität) über die Dauer von 4 Wochen nach dem Absetzen verabreicht. Für den ersten experimentellen Ansatz (**Kapitel 4**) wurden Digestaproben aus Magen, Jejunum, Ileum und Colon jeweils von 32, 39, 46 und 53 Tage alten Ferkeln genommen. Sie wurden verwendet, um bakterielle Zellzahlen und bakterielle Metaboliten *in vivo* zu ermitteln. Die größten Einflüsse der Hochzinkdiät konnten bei den 32 Tage alten Ferkeln vor allem im Magen und Jejunum beobachtet werden. Die Zellzahlen der Enterobakterien, der *Escherichia* Gruppe sowie der *Lactobacillus* spp. und von drei häufigen Spezies der *Lactobacillus* Gruppe wurden signifikant reduziert. In Hinblick auf die gesamte Versuchsperiode konnte festgestellt werden, dass der Einfluss von ZnO sich nachhaltig auf die meisten *Lactobacillus* Arten, jedoch nur sehr kurzfristig auf die Gruppe der Enterobakterien ausgewirkt hat. Keinen Einfluss schien die Verabreichung auf

Bifidobakterien, Enterokokken, Streptokokken, *Weissella* spp. und *Leuconostoc* spp. sowie auf die *Bacteroides-Prevotella-Porphyromonas* Gruppe genommen zu haben.

Von den mikrobiellen Metaboliten stieg im proximalen Verdauungstrakt Acetat im Verhältnis gesehen an und der Anteil an Propionat wurde verringert. Geringere Laktatkonzentrationen konnten in der Hochzinkgruppe in allen Tieren festgestellt werden. Allerdings verringerten sich die Unterschiede zwischen den Fütterungsgruppen hinsichtlich der mikrobiellen Metaboliten mit zunehmendem Alter der Tiere.

Für den zweiten experimentellen Ansatz (**Kapitel 5**) wurden Digestaproben aus Magen und Jejunum von 32, 39, 46 und 53 Tage alten Tieren (n = 6 pro Gruppe) genommen und unter anaeroben Bedingungen in ein Komplexmedium mit unterschiedlichen Zinkoxidkonzentrationen (80, 40, 20 und 0 µg mL^{-1}) überführt und für 16 h inkubiert. Die spezifische Wachstumsrate, das maximale Wachstum und die Anlaufzeiten wurden anhand der erhaltenen Wachstumskurven ermittelt. Die höchsten Wachstumsraten bei niedrigster Anlaufzeit konnten in den Proben der Niedrigzinkgruppe im Medium ohne Zink beobachtet werden. Die Proben der Hochzinkgruppe zeigten die höchsten Wachstumsraten und geringsten Anlaufzeiten im Medium mit gelöstem Zink. Grundsätzlich konnte beobachtet werden, dass das bakterielle Wachstum aus Proben der Hochzinkgruppe weniger stark von ZnO beeinträchtigt war und sich schneller erholte als Proben der Niedrigzinkgruppe. Auch konnte beobachtet werden, dass in Zinkmedien die Proben von älteren Tieren eine geringere Wachstumshemmung erfuhren. Die bakterielle Zellzahlanalyse mittels qPCR und einem ausgewählten Primerset konnte zeigen, dass vor allem Laktobazillen stark durch die Zinkverabreichung verringert wurden. Bifidobakterien und Enterobakterien zeigten hingegen einen schwachen Anstieg der Zellzahlen in Proben der Hochzinkgruppe. Enterobakterien zeigten erhöhte Zellzahlen in Proben aus der Hochzinkgruppe, wenn diese im Zinkmedium inkubiert wurden. Keine Veränderungen konnten hingegen bei den untersuchten Clostridien und bei der *Bacteroides-Prevotella-Porphyromonas* Gruppe gefunden werden.

Die Studien konnten zeigen, dass der Einsatz von hohen Zinkdosen im Futter zu einem reduzierten bakteriellen Wachstum unter *ex vivo* Bedingungen aus Magen- und Jejunumproben führt und darüber hinaus dauerhafte Einflussnahme auf die Struktur und Metaboliten der intestinalen Mikrobiota entlang des gesamten Verdauungstrakts nehmen kann. In Hinblick auf die starke Anpassungsfähigkeit der Mikroorganismen sowohl unter *in vitro* als auch unter *in vivo* Bedingungen auf ZnO sollte eine Verabreichung auf die ersten

Wochen nach dem Absetzen der Ferkel beschränkt sein. In **Kapitel 6** werden die Ergebnisse beider Versuche diskutiert und zusammengefasst. Dabei wird auch der Versuch unternommen, einen Beitrag zu neuen Fütterungsstrategien in der modernen Tierernährung in Hinblick auf das Mikrobiom im gastrointestinalen Trakt zu leisten.

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Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

Berlin, 6. November 2013 Ingo Starke